Proteolytic activation and solubilization of endoplasmic-reticulum cyclic AMP phosphodiesterase activity

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Dithiothreitol led to the activation and solubilization of the cyclic nucleotide phosphodiesterase activities associated with the smooth and various rough subfractions of rat liver endoplasmic reticulum. The activity in each of the subfractions exhibited somewhat different time courses, and sensitivities to dithiothreitol concentration, in respect of their solubilization and activation. Both activation and solubilization by dithiothreitol could be blocked by either thiol proteinase inhibitors or excess bovine serum albumin. Freeze–thaw solubilization was not blocked by the thiol proteinase inhibitor antipain and did not lead to the activation of the enzyme. After dithiothreitol-induced solubilization, all of the enzymes exhibited non-linear Lineweaver–Burk plots indicative of apparent negative co-operativity. In contrast, after freeze–thaw solubilization the enzyme in the smooth-endoplasmic-reticulum-plus-Golgi fraction still obeys Michaelis kinetics, as does the membrane-bound enzyme. It is possible to mimic the action of dithiothreitol in solubilizing and activating the enzyme by limited proteolysis with trypsin. Triton X-100 is highly efficient at solubilizing these enzymes, yet has little effect on their activities. Charged detergents exhibit highly selective effects on the enzymes as regards their solubilization and activity expressed.

Cyclic AMP acts as a second messenger for a variety of different hormones. The only known mechanism for its degradation is by the action of cyclic AMP phosphodiesterases. These enzymes exist in multiple forms in the cell, associated with both membrane and cytosol fractions (Appleman *et al.*, 1973; Wells & Hardman, 1977). Whereas a number of soluble species have been defined, relatively little is known about the properties of the membrane-bound species and their interaction with the membrane. They do, however, appear to exist in both peripheral and integral forms (Marchmont & Houslay, 1980; Cercek & Houslay, 1982). In the liver two distinct membrane-bound species are regulated by the action of glucagon and insulin (Houslay *et al.*, 1983*a,b*). One is a peripheral protein associated with the plasma membrane and the other is associated with an internal membrane structure. We have described distinct cyclic AMP phosphodiesterase activities associated with various

subfractions of the endoplasmic reticulum (Cercek *et al.*, 1983) and suggested that they may be due to different enzyme species. Here we show that these enzymes can be stimulated and solubilized by the action of an endogenous thiol-activated proteinase. This action can be mimicked by limited proteolysis with trypsin.

Materials and methods

These are described in the previous paper (Cercek *et al.*, 1983), with the exception of the methods described below.

Dithiothreitol treatment

Membrane fractions (1 mg of protein/ml) were incubated at 4°C with or without dithiothreitol in 0.25 M-sucrose/20 mM-Tris/HCl buffer, pH 7.4, for the time specified. At various time intervals samples were vortex-mixed, assayed for total phosphodiesterase activity with 1 μ M-cyclic AMP and then centrifuged at 165 000 $g \times 40$ min at 6°C. The supernatant was then assayed for solubilized activity. In some instances proteinase inhibitors were also added.

Abbreviations used: Tos-Arg-OMe, *N* $^{\alpha}$ -(*p*-tosyl)-arginine methyl ester; PMSF, phenylmethanesulphonyl fluoride; BSA, bovine serum albumin; CTAB, cetyltrimethylammonium bromide.

Trypsin treatment

Membrane fractions (1mg of protein/ml) were incubated at 4°C with 1.3 µg of trypsin/ml in 0.25 M-sucrose/20 mM-Tris/HCl buffer, pH 7.4, for the times indicated. Reactions were then stopped by adding an excess of trypsin inhibitor (6.1 mg/ml). Total activity and activity solubilized was determined as above.

Hypo-osmotic shock

Membranes were resuspended in 1 mM-EDTA/10 mM-Tris/HCl buffer, pH 7.5, at 4°C as described by Loten *et al.* (1978, 1980) before centrifugation at 100 000 g × 1 h at 4°C.

Antipain, leupeptin, Tos-Arg-OMe, PMSF, trypsin, soya bean trypsin inhibitor, BSA, benzamide,

CTAB, and dithiothreitol were from Sigma (U.K.) Ltd.

Results

Treatment with dithiothreitol (2 mM) led to a time-dependent activation of the phosphodiesterase activity in all of the endoplasmic-reticulum sub-fractions (Fig. 1). The RI and RII fractions were most rapidly activated, with maximal effects exerted in about 2–3 h at 4°C. This led to a 60–80% increase in these instances (Fig. 1). The activation of the RIII and smooth-plus-Golgi subfractions [for details of these subfractions, see the preceding paper (Cercek *et al.*, 1983)] occurred at a somewhat lower rate, with maximal activations of around 60% being

Fig. 1. Time-dependent activation and solubilization of endoplasmic-reticulum cyclic AMP phosphodiesterases by dithiothreitol

Membranes were incubated at 4°C for the stated periods of time with 2 mM-dithiothreitol, after which cyclic AMP phosphodiesterase activity was determined at 1 µM-cyclic AMP, using the entire sample, to determine the extent of activation (●) and a 100 000 g × 20 min supernatant was prepared, to determine the degree of solubilization (□). In the absence of dithiothreitol no changes in enzyme activity or release occurred over the times studied. Total activities are expressed as percentages of those observed in the absence of dithiothreitol. (a) RI; (b) RII; (c) RIII; (d) smooth-plus-Golgi fraction.

Fig. 2. Dose-dependency of the effects of dithiothreitol on endoplasmic-reticulum cyclic AMP phosphodiesterase. Membranes were incubated for 20 h at 4°C in the presence of various concentrations of dithiothreitol. After this time both the total cyclic AMP phosphodiesterase activity (●) and the percentage solubilized (see the legend to Fig. 1) (□) were determined. In control experiments without dithiothreitol, there was no change in phosphodiesterase activity or release of activity over this time. Total activities are expressed as percentages of that observed in the absence of dithiothreitol. (a) RI; (b) RII; (c) RIII; (d) smooth-plus-Golgi fraction.

achieved after about 20 h exposure (Fig. 1). As well as activating these enzymes, dithiothreitol also initiated the release of these enzymes from their respective membrane fractions. There appeared to be no close correlation between activation and solubilization (Fig. 1).

Both the release of enzyme from the fractions and the degree of activation elicited was dependent on the concentration of dithiothreitol used (Fig. 2). In all cases solubilization tended to reach a plateau at high dithiothreitol concentrations. We also note that high dithiothreitol concentrations led to an inhibition of activity. From observation of the data in Fig. 2, it would appear that the concentration of dithiothreitol exerting half-maximal activating effects was less than the concentration required to achieve half-maximal solubilizing effects. Furthermore the activities in each fraction appeared to show somewhat different sensitivities to the effect of dithiothreitol.

If incubations with dithiothreitol were carried out with various proteinase inhibitors present we observed that the activation was blocked by antipain and leupeptin. In contrast Tos-Arg-OMe, PMSF and soya-bean trypsin inhibitor had no effect (Table 1).

Also if excess BSA was present, then this too prevented activation. Some of these agents, when added in the absence of dithiothreitol, exerted selective effects on the various phosphodiesterase activities (Table 1). We also see (Table 2) that both antipain and leupeptin prevented the solubilization of the enzymes in all of the fractions. In contrast soya-bean trypsin inhibitor and benzamide hydrochloride had little effect on the amount of enzyme solubilized. Tos-Arg-OMe and PMSF had no effect on the solubilization of RIII activity, whereas bovine serum albumin inhibited the release (results not shown).

Activity can be released from the membrane by repeated freeze-thawing (Cercek *et al.*, 1983) in a fashion that does not lead to activation in any fraction (<5% change). This release is not inhibited by the presence of antipain (results not shown). Indeed, when antipain was present during the preparation of these fractions it had no effect on either the yield of phosphodiesterase activity or its distribution in the various fractions (results not shown). The kinetics of both the freeze-thawed solubilized enzymes and their dithiothreitol-solubilized counterparts are summarized in Table 3.

Table 1. *Action of proteinase inhibitors on the dithiothreitol-mediated activation of endoplasmic-reticulum cyclic AMP phosphodiesterase activity*

Incubations were carried out at 4°C for 24 h at a membrane protein concentration of 1 mg/ml. Results are means of three experiments $\pm$ S.D. Results given in parentheses are activities in the absence of dithiothreitol.

Additions	Fractions ...	Activity (%)			
		Smooth plus Golgi	RIII	RII	RI
No dithiothreitol		(100)	(100)	(100)	(100)
2 mM-Dithiothreitol alone		136 $\pm$ 1	151 $\pm$ 8	160 $\pm$ 6	150 $\pm$ 7
+ Antipain (100 μ M)		101 $\pm$ 9 (100 $\pm$ 8)	102 $\pm$ 4 (94 $\pm$ 4)	106 $\pm$ 7 (95 $\pm$ 2)	106 $\pm$ 5 (90 $\pm$ 4)
+ Leupeptin (100 μ M)		79 $\pm$ 2 (75 $\pm$ 4)	86 $\pm$ 4 (78 $\pm$ 5)	100 $\pm$ 1 (97 $\pm$ 3)	91 $\pm$ 7 (102 $\pm$ 8)
+ BSA (25 mg/ml)		84 $\pm$ 2 (70 $\pm$ 7)	71 $\pm$ 8 (70 $\pm$ 5)	97 $\pm$ 8 (70 $\pm$ 4)	95 $\pm$ 10 (82 $\pm$ 2)
+ Benzamidine hydrochloride (50 mM)		139 $\pm$ 2 (110 $\pm$ 3)	128 $\pm$ 15 (115 $\pm$ 5)	115 $\pm$ 5 (116 $\pm$ 1)	117 $\pm$ 7 (94 $\pm$ 1)
+ Tos-Arg-OMe (2 mM)		133 $\pm$ 6 (107 $\pm$ 6)	153 $\pm$ 8 (106 $\pm$ 13)	127 $\pm$ 8 (85 $\pm$ 5)	132 $\pm$ 9 (79 $\pm$ 2)
+ PMSF (1 mM)		131 $\pm$ 5 (105 $\pm$ 8)	162 $\pm$ 2 (108 $\pm$ 9)	161 $\pm$ 5 (117 $\pm$ 3)	144 $\pm$ 5 (107 $\pm$ 6)
+ Soya-bean trypsin inhibitor (1 mg/ml)		175 $\pm$ 3 (90 $\pm$ 1)	150 $\pm$ 10 (89 $\pm$ 5)	165 $\pm$ 4 (102 $\pm$ 4)	156 $\pm$ 5 (87 $\pm$ 8)

Table 2. *The action of various proteinase inhibitors on the dithiothreitol-mediated solubilization of endoplasmic-reticulum phosphodiesterases*

Membranes (1 mg of protein/ml) were incubated with the various ligands for 20 h at 4°C, after which they were centrifuged at 100 000 $g \times 20$ min in a Beckman Airfuge. Results are means of three experiments $\pm$ S.D.

Additions	Fractions ...	Activity solubilized (%)			
		Smooth plus Golgi	RIII	RII	RI
Dithiothreitol (2 mM)		25 $\pm$ 5	61 $\pm$ 5	60 $\pm$ 2	69 $\pm$ 9
+ Antipain (100 μ M)		7 $\pm$ 3	13 $\pm$ 7	5 $\pm$ 1	12 $\pm$ 2
+ Leupeptin (100 μ M)		10 $\pm$ 1	16 $\pm$ 1	12 $\pm$ 3	14 $\pm$ 3
+ Benzamidine hydrochloride (50 mM)		27 $\pm$ 4	56 $\pm$ 2	59 $\pm$ 7	42 $\pm$ 7
+ Soya-bean trypsin inhibitor (1 mg/ml)		25 $\pm$ 12	64 $\pm$ 7	58 $\pm$ 6	69 $\pm$ 3
+ BSA (25 mg/ml)		15 $\pm$ 5	8 $\pm$ 2	15 $\pm$ 4	16 $\pm$ 2

Table 3. *Kinetic parameters for the solubilized cyclic AMP phosphodiesterase activity from endoplasmic reticulum*

Freeze-thaw solubilized enzyme was prepared as in the Materials and methods section. The dithiothreitol-solubilized enzyme was prepared by taking the supernatant from membranes (1 mg of protein/ml) incubated with 2 mM-dithiothreitol for 20 h at 4°C before centrifugation at 100 000 $g \times 1$ h. The range of substrate concentrations employed was as described in the preceding paper (Cercek *et al.*, 1983). Results are means $\pm$ S.D. of three experiments.

	Fraction	K_m^1 (μ M)	K_m^2 (μ M)	V_{max} [(pmol $\cdot$ min $^{-1}$ $\cdot$ mg of protein) $^{-1}$]	
				V_{max}^1	V_{max}^2
Freeze-thaw solubilized	Smooth plus Golgi	8.6 $\pm$ 3.1	—	400 $\pm$ 25	—
	RIII	0.64 $\pm$ 0.06	43 $\pm$ 13	84 $\pm$ 5	734 $\pm$ 94
	RII	0.77 $\pm$ 0.33	27 $\pm$ 5	110 $\pm$ 5	667 $\pm$ 10
	RI	1.7 $\pm$ 0.5	40 $\pm$ 5	158 $\pm$ 21	1067 $\pm$ 38
Dithiothreitol-solubilized	Smooth plus Golgi	1.5 $\pm$ 0.5	10 $\pm$ 4	222 $\pm$ 12	714 $\pm$ 85
	RIII	0.8 $\pm$ 0.1	26 $\pm$ 5	202 $\pm$ 3	939 $\pm$ 99
	RII	0.5 $\pm$ 0.1	53 $\pm$ 13	198 $\pm$ 18	1602 $\pm$ 91
	RI	1.9 $\pm$ 0.9	40 $\pm$ 19	325 $\pm$ 106	1333 $\pm$ 94

Fig. 3. Action of limited trypsin proteolysis on endoplasmic-reticulum cyclic AMP phosphodiesterase activity and solubilization

Fractions were incubated at 4°C with trypsin, as described in the Materials and methods section, for various periods of time before stopping the reaction with trypsin inhibitor. Both the total activity (●) and degree of solubilization (□) were determined. (a) RI; (b) RII; (c) RIII; (d) smooth-plus-Golgi fraction.

Table 4. Detergent solubilization of endoplasmic-reticulum cyclic AMP phosphodiesterase activity

Membranes (1mg of protein/ml) were incubated for 30min at 4°C before assay. They were also centrifuged at 100000g×1h before assay for supernatant activity to determine the degree of solubilization. Results are means ± s.d. (n=3). Abbreviation used: ND, not determined.

Detergent	Fraction ...	Smooth plus Golgi	RIII	RII	RI
Triton X-100 (1%)	Activity (% of native activity)	112 ± 4	93 ± 5	94 ± 3	95 ± 4
	Solubilization (%)	100 ± 12	66 ± 3	80 ± 7	75 ± 15
Sodium cholate (2%)	Activity (% of native activity)	43 ± 5	34 ± 4	30 ± 2	ND
	Solubilization (%)	3 ± 3	26 ± 3	73 ± 10	ND
CTAB (0.1%)	Activity (% of native activity)	125 ± 5	112 ± 5	140 ± 4	ND
	Solubilization (%)	7 ± 1	45 ± 11	<2	ND

All of the dithiothreitol-solubilized enzymes exhibited non-linear Michaelis plots with a relatively low K_m component having a limiting K_m in the micromolar range. The limiting kinetic constants for K_m are very similar for the enzymes in the rough-endoplasmic-reticulum fractions for both the freeze-thaw-solubilized enzymes and the dithiothreitol-solubilized enzymes. In contrast the freeze-thawed solubilized enzyme from the smooth-endoplasmic-reticulum fraction exhibits linear Michaelis kinetics like the membrane-bound enzyme (Cercek *et al.*, 1983), albeit with a somewhat lower K_m . By using low concentrations of trypsin it was possible to elicit both the activation and solubilization of enzyme activity in these fractions

(Fig. 3). There was no clear correlation between the time course of activation and release of the enzymes. Degrees of activation of the order of 30–50% were achieved with levels of solubilization of 10–30% (Fig. 3). The enzymes associated with the various fractions could also be solubilized using detergents. Table 4 shows that the non-ionic detergent Triton X-100 is very efficient at solubilizing the activities in all of the various fractions. It achieves this while eliciting little change in the total enzyme activity in any particular fraction, except that in the smooth-plus-Golgi fraction it is slightly activated. The anionic detergent sodium cholate was found to inhibit enzyme activity, whereas the cationic detergent CTAB elicited a

modest activation. The degree of solubilization elicited by these various charged detergents varied widely between the enzymes in the different fractions. Hypo-osmotic shock (see Loten *et al.*, 1978, 1980) did not lead to the activation or solubilization of the enzymes (results not shown).

Discussion

Dithiothreitol and other thiol-protective reagents find common use in purification schemes and assays of many enzymes, including cyclic AMP phosphodiesterase (see, e.g., Donnelly, 1978; Ilien *et al.*, 1978; Klee & Krinks, 1978; Erneux *et al.*, 1980; Sharma *et al.*, 1980; Martins *et al.*, 1982). We show here that exposure of endoplasmic-reticulum subfractions to dithiothreitol led to the activation and solubilization of the endogenous cyclic AMP phosphodiesterase activities. This activation and solubilization would appear to be elicited by the stimulation of a thiol proteinase by dithiothreitol, as both effects could be specifically blocked by either antipain or leupeptin. These agents are known to inhibit thiol proteinases (Umezawa, 1976), whereas other proteinase inhibitors exerted little effect (Tables 1 and 2). Consistent with this interpretation were our observations that limited trypsin proteolysis could mimic the effect of dithiothreitol. Furthermore, high concentrations of serum albumin were shown to block this effect, presumably by a competitive action. It would seem unlikely, however, that these phosphodiesterase enzymes are necessarily significantly acted upon by these proteinases *in vivo*, as cytosolic proteins would presumably act as competing substrates. In this respect we noted that preparation of the subfractions in the presence of antipain gave similar yields, distribution and specific activities for the enzyme activities in the various subfractions. The source of the proteinase exerting these effects could be either the endoplasmic-reticulum subfractions themselves (see Sogawa & Takahashi, 1978) or lysosomal contamination, although this is low in our washed fractions (Cercek *et al.*, 1983).

Makino *et al.* (1982) have observed that the phosphodiesterase activity of a 'crude microsomal fraction' from adipocytes was also activated and solubilized by a dithiothreitol-stimulated thiol proteinase under similar conditions to those used in the present study. Also a variety of soluble cyclic AMP phosphodiesterases have been shown to be activated by limited proteolysis (see Wells & Hardman, 1977; Strewler & Manganiello, 1979).

Neither the time courses for the release and activation processes of the phosphodiesterases that occur upon exposure to dithiothreitol or low trypsin concentrations, nor the dose-response curves for these processes, show close correlation. This sug-

gests that activation does not occur simply as a result of the release of the enzymes from the membrane. Consistent with this are our observations that solubilization effected by freeze-thawing, a process not involving proteolysis, as it is unaffected by antipain, does not lead to enzyme activation. Furthermore the enzymes can be efficiently solubilized by the non-ionic detergent Triton X-100, without activating the phosphodiesterase activity in all of these various fractions (Table 4). Interestingly the cationic detergent sodium cholate solubilized the enzymes while inhibiting them, whereas the anionic detergent CTAB actually activated the enzymes while effecting little solubilization (Table 4). Presumably these observations reflect charge effects on the protein. Thus the activation of the enzyme by either dithiothreitol or trypsin is probably as a result of limited proteolysis rather than solubilization in itself.

Unlike the enzymes in the other fractions, which are integral proteins (Cercek *et al.*, 1983), that in the RI fraction is a peripheral protein, yet it was still released and activated by limited proteolysis. It is possible that it binds to an integral protein in the membrane (for discussion see Cercek *et al.*, 1983). Thus release of the phosphodiesterase might occur by the proteolytic cleavage of the site interacting with this integral (binding) protein.

The time courses and dose-response curves for solubilization and activation of the phosphodiesterase activities (Figs. 1–3) were somewhat dissimilar for the various enzyme activities, which again supports our contention (Cercek *et al.*, 1983) that rather different enzymes are responsible for each of these activities. Indeed we also note (Table 1) that some of the proteinase inhibitors, e.g. Tos-Arg-OMe and leupeptin, exerted selective inhibitory effects on the various phosphodiesterase activities. Furthermore, the dithiothreitol-elicited proteolysis of the enzyme from the smooth-plus-Golgi fraction has a pronounced effect on the kinetics of this enzyme (Table 3). This change is not merely a consequence of solubilization, as the freeze-thaw solubilized enzyme still obeys normal Michaelis kinetics.

Makino *et al.* (1982) have claimed that, in adipocytes, the phosphodiesterase activity solubilized by dithiothreitol is the insulin-stimulated enzyme. However, we have shown that microsomes from liver (Cercek *et al.*, 1983) or hepatocytes (S. R. Wilson, unpublished work) contain a number of rather different phosphodiesterase activities. It is therefore likely that the crude microsomal fraction used by Makino and co-workers actually contained a heterogeneous selection of phosphodiesterase enzymes. In liver there are two specific membrane-bound cyclic AMP phosphodiesterases that are activated by insulin (for review see Houslay *et al.*, 1983b). One is associated with the plasma membrane (Houslay & Marchmont, 1981) and the other

with an internal-membrane fraction (Houslay *et al.*, 1983b). The internal-membrane enzyme (Loten *et al.*, 1978) is distinct from the various activities in these endoplasmic-reticulum fractions (Cercek *et al.*, 1983) on the basis of kinetic and physical properties as well as thermostability. However, this insulin-stimulated enzyme can be solubilized and activated by limited proteolysis (Loten *et al.*, 1980). Loten *et al.* (1980) demonstrated that solubilization and activation could be effected merely by hypo-osmotic shock and was blocked by benzamidine (30–50 mM). This contrasts with the various microsomal enzymes that are not activated or solubilized by hypo-osmotic shock and where benzamidine does not prevent dithiothreitol-elicited solubilization (Table 2). We note, however, that benzamidine did exert some inhibitory effect (Table 1) in the dithiothreitol-mediated activation of the enzymes in the various rough fractions but not in the smooth-plus-Golgi fraction. The proteolytic sensitivity of microsomal cyclic AMP phosphodiesterases provides further evidence that these microsomal enzymes are distinct from the insulin-stimulated phosphodiesterase (Loten *et al.*, 1978, 1980) associated with an internal membrane fraction. Indeed insulin did not activate these microsomal phosphodiesterase activities after either exposure of intact hepatocytes to insulin or exposure of the fractions to insulin (S. R. Wilson & M. D. Houslay, unpublished work).

These experiments emphasize the need for caution in the use of thiol-protective reagents in investigating phosphodiesterase activity in cells and in studies attempting to assess enzyme distribution in particulate and soluble fractions (see, e.g., Appleman *et al.*, 1973; Wells & Hardman, 1977; Thompson & Strada, 1978). It is possible that a significant fraction of so-called soluble activity could accrue from the release of various membrane-bound species by limited proteolysis, sonication, hypo-osmotic shock (breaking lysosomes), freeze-thawing and other procedures. Our studies thus highlight the complexity of membrane phosphodiesterases associated in rather different ways with a variety of cellular membranes.

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