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Reduction of protein disulfide bonds in an oxidizing environment

The disulfide bridge of cholera toxin A-subunit is reduced in the endoplasmic reticulum

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Abstract Following retrograde transport to the endoplasmic reticulum (ER) the A-subunit of cholera toxin (CTX-A) is partially cleaved into CTX-A1 and CTX-A2 by reduction of a disulfide bridge [Majoul et al. (1996) J. Cell Biol. 133, 777–789], although the redox state in the ER favors disulfide formation. We show here that the disulfide bridge of CTX-A is cleaved in vitro already at GSH/GSSG ratios between 1 and 3. Protein disulfide isomerase (PDI) exerts only a minor accelerating effect. Various mixed disulfide intermediates (CTX-A1-S-S-CTX-A1; PDI-S-S-A2; PDI-S-S-A1) appear during CTX-A reduction. These results indicate that in the ER protein disulfide formation and protein disulfide reduction can take place simultaneously.

Key words: Cholera toxin; Protein disulfide isomerase; Endoplasmic reticulum; Glutathione; Redox state; Protein disulfide

1. Introduction

Apart from glycosylation disulfide bond formation is the most prominent post-translational modification in secretory proteins. Disulfide bond formation already begins co-translationally. It is catalyzed by protein disulfide isomerase and most likely also by other members of the family of thioredoxin-like proteins in the endoplasmic reticulum (ER). Disulfide bond formation in nascent proteins requires an oxidizing redox environment. The existence of such an environment in the endoplasmic reticulum has been convincingly shown by Hwang et al. [1], who calculated GSH/GSSG ratios for the ER in the range from 1 to 3. Protein disulfide isomerase, like most other known soluble resident ER-proteins, possesses a C-terminal tetrapeptide retention signal (KDEL for protein disulfide isomerase (PDI) from mammalian cells). As first shown by Pelham's group [2–6], this peptide represents a retrieval rather than a retention signal: resident soluble ER proteins which escape into the Golgi interact in this compartment with a KDEL receptor, which is subsequently transported back to the ER together with its ligand where the KDEL protein becomes released from its receptor. The receptor recycles back to the Golgi.

We have recently introduced cholera toxin (CTX), whose A-subunit contains a C-terminal KDEL sequence, as a tool to study the back-transport of KDEL proteins from the Golgi to the ER [7]. We could demonstrate that the cholera toxin A-subunit (CTX-A), but not the B-subunit is transported back from the Golgi to the ER, most likely by retrograde vesicular transport. The A-subunit of cholera toxin is proteolytically cleaved by *Vibrio cholerae* between Arg^{192} and Ser^{193}. The resulting A1- and A2-subunits remain linked together by a disulfide bridge (Fig. 1). The A1-subunit (CTX-A1) catalyzes the ADP-ribosylation of the α-subunit of the hetero-trimeric G_s protein, and thus represents the toxic principle. We have observed recently that CTX-A is partially reduced to form CTX-A1- and A2-subunits after its arrival in the endoplasmic reticulum. This could be demonstrated even under conditions where the cells were homogenized and fractionated in the presence of NEM [7]. As this finding seems to be in conflict with the strongly oxidizing redox state in the ER, we have analyzed the reduction of CTX-A in the presence of different GSH/GSSG ratios in the absence and presence of protein disulfide isomerase. The results indicate that CTX-A and possibly other cellular protein disulfides can be reduced in the ER in spite of the oxidizing conditions prevailing in this compartment.

2. Materials and methods

2.1. Materials

Cholera toxin and cholera toxin A-subunit were purchased from Calbiochem/Novabiochem, Bad Soden, Germany. Different batches of CTX-A contained different amounts of CTX-A which was not

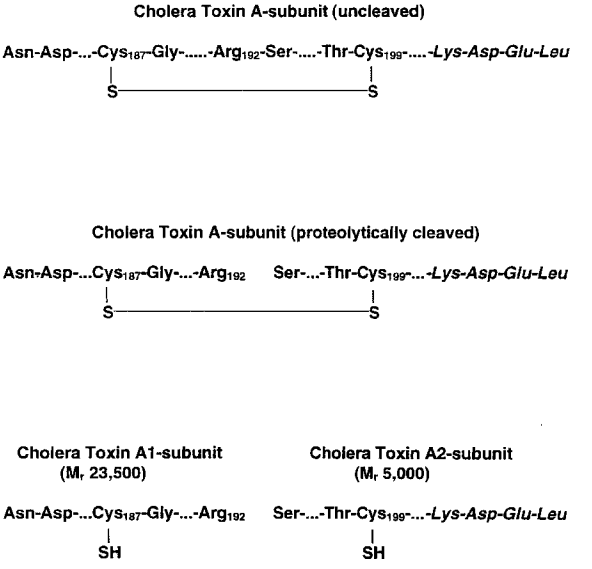

Fig. 1. Primary structure of cholera toxin A-subunit and its cleavage products CTX-A1 and CTX-A2.

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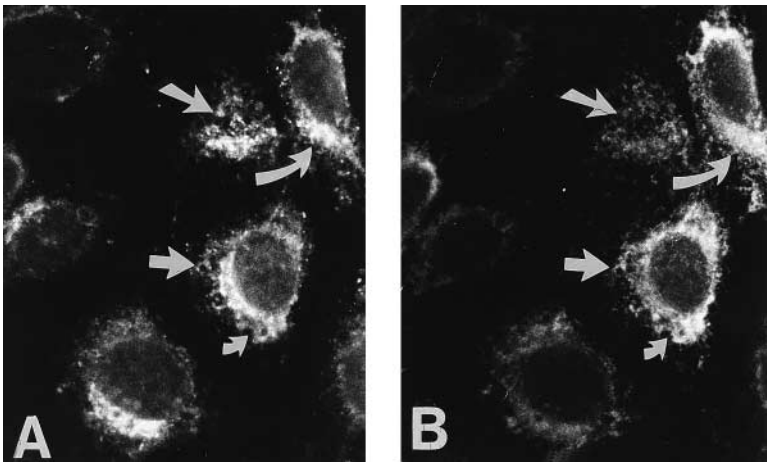
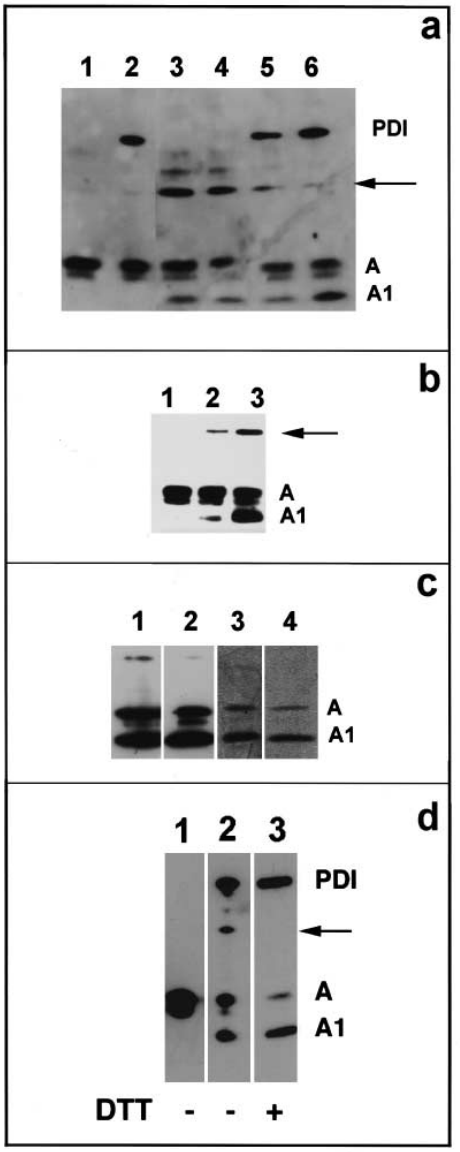


Fig. 2. Colocalization of CTX-A (A) and PDI (B) in Vero cells 90 min after initiation of uptake of CTX. Examples of colocalization of CTX-A and PDI are marked by arrows. Double immunofluorescence using a guinea-pig anti-CTX-A antiserum and a rabbit anti-bovine PDI antiserum. Although some CTX-A is still existing in Golgi-like structures, CTX-A colocalizes to a large extent with the ER marker PDI.



proteolytically cleaved between Arg^{192} and Ser^{193} (see Section 3). GSH and GSSG as well as the BM chemiluminescence blotting kit came from Boehringer, Mannheim, Germany, iodixanol from Nycomed Pharma, Oslo, Norway. PDI was isolated from bovine liver microsomes according to [8] and tested for activity using the insulin reduction assay of Holmgren et al. [9] using GSH as reductant [10]. Polyclonal antibodies were raised against CTX-A, CTX-A1, and against a peptide of CTX-A2 as given in [7]. An antiserum against PDI from bovine liver was produced in rabbits.

2.2. Methods

2.2.1. Localization of PDI and CTX-A by immunofluorescence. Vero cells were incubated with 0.1 μg/ml cholera toxin and the uptake and subcellular distribution of its subunits was followed for 90 min by immunofluorescence as described previously [7]. The cells were then

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Fig. 3. In vitro formation of CTX-A1 and CTX-A2 in the presence of different GSH/GSSG ratios. a: Immunoblots following non-reducing SDS-PAGE using antibodies directed against PDI and CTX-A. Formation of CTX-A1 at molar GSH/GSSG ratios of 1/1 (lanes 4 and 5) and 3/1 (lanes 3 and 6) in the absence (lanes 3 and 4) or presence (lanes 5 and 6) of PDI. The initial concentration of CTX-A was 0.1 μM, that of PDI (monomer) 1.3 μM. The incubation was performed for 30 min at 35°C. Controls were incubated without GSH and GSSG in the absence (lane 1) or presence (lane 2) of PDI. The arrowhead indicates the position of the CTX-A1-S-S-CTX-A1 intermediate. b: Immunoblot following SDS-PAGE using an antibody against CTX-A. Comparison of the reduction of CTX-A at GSH/GSSG ratios of 10/1 (lane 2) and 100/1 (lane 3). The control (lane 1) did not contain GSH or GSSG. The incubation was performed at room temperature for 20 min. Note that even at the higher GSH/GSSG ratio a considerable amount of CTX-A remains unsplit. c: Incomplete cleavage of CTX-A following denaturation with 6 M guanidinium chloride/300 mM DTT, followed by treatment with 100 mM iodoacetate (lanes 2 and 4). The results are compared with those where CTX-A was only treated with 25 mM DTT (lanes 1 and 3). Following reducing SDS-PAGE and immunoblotting, CTX-A and CTX-A1 were reacted with a rabbit anti-CTX-A antibody. Detection was performed either by chemoluminescence (lanes 1 and 2), or using the peroxidase substrate β-chloronaphthol (lanes 3 and 4). d: Disappearence of the CTX-A1-S-S-CTX-A1 disulfide following addition of DTT. CTX-A was incubated with GSH/GSSH at a ratio of 2/1 in the presence of PDI for 30 min at 35°C (lane 2). An aliquot of the sample was then treated with 25 mM DTT (lane 3). The samples were analyzed by SDS-PAGE and immunoblotting using antibodies against PDI and against CTX-A. Lane 1 represents a control incubation without GSH, GSSG, and PDI. Note the disappearance of the CTX-A1 disulfide.

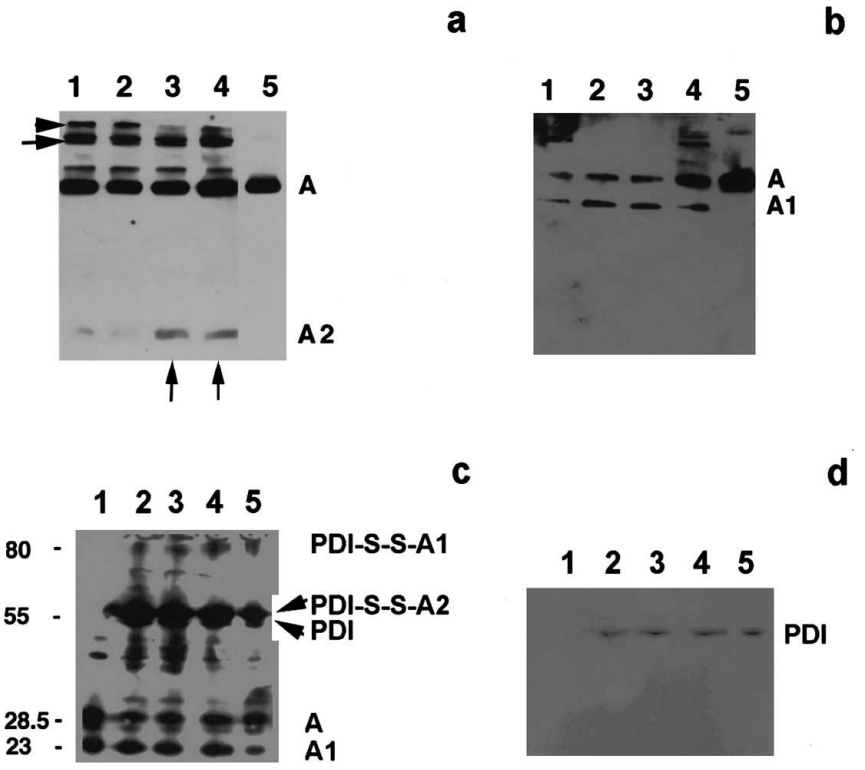

Fig. 4. Formation of mixed disulfides between PDI and CTX-A2 and PDI and CTX-A1. a, b: The incubation of CTX-A (0.3 μM) with PDI (1.3 μM) was performed for 30 min at 35°C at GSH/GSSG ratios of 1/1 (lanes 1 and 3, panels a and b) or 2/1 (lanes 2 and 4, panels a and b). Lane 5 (panels a and b) is a control incubation without GSH, GSSG, or PDI. Following incubation, aliquots of the samples depicted in lanes 1 and 2 were treated with 25 mM DTT prior to non-reducing SDS-PAGE and immunoblotting. Immunoblotting was performed with anti-PDI plus anti-CTX-A2 in panel a, or only with anti-CTX-A1 in panel b. The arrowhead indicates the position of the mixed disulfide between PDI and CTX-A2. Note that the mixed disulfide disappears and free CTX-A2 appears (panel a, lanes 3 and 4) after DTT treatment. c and d: CTX-A was incubated at a GSH/GSSG ratio of 2/1 as in panels a and b, except that the incubations were stopped after 5 min (lane 2), 15 min (lane 3), 30 min (lane 4), or 40 min (lane 5) and that a 10% instead of a 16% gel was used for non-reducing SDS-PAGE. For immunostaining an anti-CTX-A antibody (detecting CTX, CTX-A1 and CTX-A2) and an anti-PDI antibody were used in panel c, whereas in panel d PDI is depicted following silver staining. The 13-fold lower amount of CTX-A is not visible after silver staining. Note the appearance of PDI-S-S-CTX-A1. The anti-CTX-A antibody used in panel c reacts with CTX-A, CTX-A1, and CTX-A2. It gives a stronger reaction with CTX-A2 than the anti-CTX-A2-peptide antibody used in panel a. This explains why in c the band corresponding to PDI-S-S-A2 is so strong that it almost covers the PDI band detected with the PDI-specific antibody. Note that the bands corresponding to PDI-S-S-CTX-A1 and PDI-S-S-CTX-A2 do not appear in lane 1 of panel c, where the incubation was performed in the absence of PDI.

fixed in 0.2% glutaraldehyde, 2% paraformaldehyde for 10 min on ice and for 20 min at room temperature and blocked with 50 mM NH_4Cl. The cells were permeabilized with 0.15% (w/v) saponin in PBS/0.2% gelatin. For double immunofluorescence a primary guinea pig anti-CTX-A antibody and a primary rabbit anti-PDI antibody were used. Epifluorescence was detected using an Axiovert microscope with a Plan-Neofluar ×100/1.30 objective.

2.2.2. Reduction of CTX-A in vitro. Unless otherwise mentioned 8.5 μg/ml CTX-A was incubated at the indicated temperatures in buffer A (100 mM potassium phosphate, 1 mM EDTA, pH 7.0) in the presence of different GSH/GSSG ratios in the absence or presence of 1.3 μM PDI corresponding to a molar PDI (monomer)/CTX-A ratio of 13/1. The total concentration of GSH+GSSG was always fixed at 5 mM. All assays were stopped by the addition of NEM to a final concentration of 10 mM. Laemmli mix [11] without 2-mercaptoethanol was added to aliquots from the incubation mixtures and heated for 5 min at 90°C followed by non-reducing polyacrylamide gel electrophoresis according to [12]. The separated proteins were blotted onto nitrocellulose and the immune reactions were performed using standard procedures. Detection was done by chemiluminescence. Where indicated, immune reactions were performed simultaneously with two different antibodies (e.g. anti-PDI and anti-CTX-A).

In order to examine whether all CTX-A was proteolytically cleaved, 50 μg CTX-A was incubated with 600 μl 6 M guanidinium chloride, 300 mM DTT for 30 min at room temperature. The sample was concentrated to 100 μl by centrifugation through Centricon10 filters and 1.5 ml of solution B (100 mM iodoacetamide, 2 mM EDTA, 100 mM Tris-HCl, pH 8.0) was added followed again by centrifugal concentration to 400 μl. This step was repeated twice. The sample was concentrated to 200 μl, 1.5 ml PBS was added, and the sample concentrated to 200 μl as before. The PBS washing step was repeated twice, the sample concentrated to a final volume of 150 μl, and used for reducing SDS-PAGE and immunoblotting.

3. Results

In accordance with our previous data [7] 90 min after initiation of cholera toxin uptake CTX-A arrives in the endoplasmic reticulum as demonstrated by its partial colocalization with PDI, considered to be an ER marker protein (Fig. 2).

Hwang et al. [1] have determined GSH/GSSG ratios in the ER to be between 1/1 and 3/1. Therefore, we have examined whether CTX-A could be reduced at these GSH/GSSG ratios. In the absence of GSH and GSSG, a reduction of CTX-A did not occur, irrespective of whether PDI was present (Fig. 3a, lane 2) or not (Fig. 3a, lane 1). At a GSH/GSSG ratio of 1/1 (Fig. 3a, lanes 4 and 5) or 3/1 (Fig. 3a, lanes 3 and 6) CTX-A1 was formed indicating that reductive cleavage of some of the CTX-A had taken place. The amount of CTX-A formed was

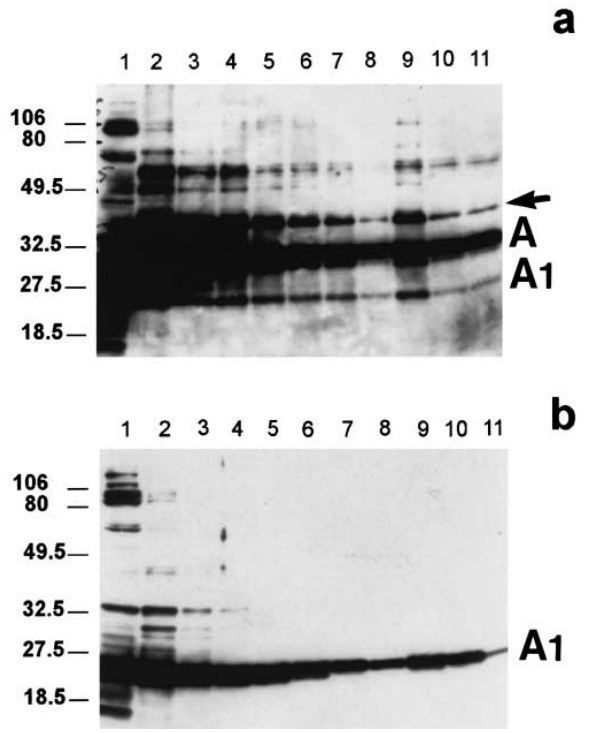

Fig. 5. Appearance of mixed disulfides in Vero cells 45 min after initiation of CTX uptake as in Fig. 3. In contrast to the experiment shown in Fig. 3, the fractions from the iodixanol gradient were directly used for SDS-PAGE according to [12] under non-reducing (a) and reducing (b) conditions without further purification by sedimentation and washing. Moreover, the material layered onto the gradient contained much more plasma membranes than in Fig. 3 as precentrifugation was carried out at 1000×*g* and not at 3000×*g*. The arrow marks the CTX-A1 disulfide; (A) = CTX-A; (A1) = CTX-A1.

higher at a GSH/GSSG ratio of 3/1 than at a ratio of 1/1. Interestingly, CTX-A1-S-S-CTX-A1 appeared as an intermediate (arrow in Fig. 3a). The additional presence of PDI had only a minor additional effect on CTX-A cleavage, but the appearance of the CTX-A1-S-S-CTX-A1 intermediate was decreased. The CTX-A1-S-S-CTX-A1 species was identified by the following criteria: (1) it runs with an approximate molecular mass of 45 kDa (monomeric CTX-A1 has a molecular mass of about 23.5 kDa), (2) it reacts with an antibody directed against CTX-A1, (3) the band disappears after treatment of the sample with DTT (Fig. 3d). Although reductive cleavage of CTX-A was already observed at a GSH/GSSG ratio of 1/1, some uncleaved CTX-A remained even at a GSH/GSSG-ratio of 100/1 (Fig. 3b, lane 3). This would be difficult to explain if all CTX-A molecules were proteolytically split and CTX-A1 and CTX-A2 were only linked by a disulfide bridge. We therefore suspected that some of the CTX-A represented CTX-A which had escaped proteolytic cleavage by *V. cholerae* (see Fig. 1) and thus would not change its position in the gel after cleavage of the disulfide bond. In order to test this possibility, we treated CTX-A with 6 M guanidinium chloride in the presence of 300 mM DTT and subsequently with iodoacetamide to block any reoxidation of -SH groups. Even after this treatment a significant amount of CTX-A remained (Fig. 3c) indicating that indeed a part of the CTX-A used in these experiments had not been proteolytically cleaved.

While the appearance of the CTX-A1-S-S-CTX-A1 species is decreased in the presence of PDI+GSH/GSSG as compared to GSH/GSSG alone, mixed disulfides between PDI and CTX-A2 and between PDI and CTX-A1 can be observed. In the presence of PDI, the PDI-S-S-CTX-A2 species is observed as a thin band which migrates slightly more slowly than PDI (Fig. 4a, lanes 1 and 2). This band disappears after treatment of the samples with DTT (Fig. 4a, lanes 3 and 4) and a band of low molecular mass (about 5 kDa) appears which reacts with an antibody specific for CTX-A2 (Fig. 4a, lanes 3 and 4). Neither the PDI-S-S-CTX-A2 band nor CTX-A2 can be observed if a CTX-A1-specific antibody is used (Fig. 4b), indicating that indeed the high molecular mass band which had disappeared and the low molecular mass band which newly appeared (Fig. 4a) represented specifically CTX-A2. When 10% instead of 16% gels were used, the mixed PDI-S-S-CTX-A1 disulfide could also be demonstrated (Fig. 4c), which disappeared after treatment with DTT (not shown here). The fact that the two mixed disulfides (PDI-S-S-CTX-A1 and PDI-S-S-CTX-A2) did not appear in the absence of PDI (Fig. 4c, lane 1) further supports the identity of the corresponding bands in lanes 2–5 of Fig. 4c.

Mixed disulfides of CTX-A could also be observed in intact Vero cells treated with CTX (Fig. 5). It is clearly visible that under reducing conditions the various CTX-A positive fractions are mainly converted to CTX-A1, indicating the existence of CTX-A1 containing mixed disulfides (in particular CTX-A1-S-S-CTX-A1) under non-reducing conditions.

The time dependence of the reduction of CTX-A at a GSH/GSSG ratio of 2/1 is shown in Fig. 6. In the absence of PDI, only a trace amount of CTX-A1 became visible after 2 min, whereas in the presence of PDI the formation of CTX-A1 could already clearly be seen at this time point. The difference is not due to different loading conditions as the initial acceleration of the reduction of CTX-A was observed in several experiments. After 6 min, the formation of CTX-A1 had increased to the same extent in the absence or presence of PDI. When the incubation was continued up to 10 min, only a moderate further increase in the formation of CTX-A1 occurred, and an additional effect of PDI was no longer detectable. These results indicate that the final redox equilibrium is

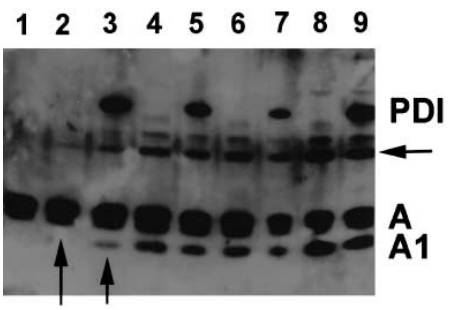

Fig. 6. Time-dependent reduction of CTX-A in the absence (lanes 1, 2, 4, 6, and 8) or presence (lanes 3, 5, 7, and 9) of PDI. CTX-A was incubated at 35°C (as given in the legend to Fig. 4a) for 2 min (lanes 2 and 3), 6 min (lanes 4 and 5), 8 min (lanes 6 and 7), and 10 min (lanes 8 and 9). Lane 1 is a control incubation which did not contain GSH, GSSG, or PDI. At the times given, incubations were stopped by addition of NEM and the samples were processed by non-reducing SDS-PAGE and immunoblotting using anti-CTX-A antibodies and an antibody against PDI. Note the slight accelerating affect of PDI (lanes 2 and 3) which can no longer be observed at later time points.

reached within a few minutes, and that PDI accelerates this process only to a minor degree.

4. Discussion

The results show clearly that the reduction of a protein disulfide can occur under the relatively oxidizing conditions existing in the ER: a partial cleavage of CTX-A into CTX-A1 and CTX-A2 can already be observed at a GSH/GSSG ratio of 1. The reduction of CTX-A can occur even in the absence of PDI. PDI catalyzes only a slight acceleration of CTX-A reduction under these conditions. In the absence of PDI the reduction of CTX-A is accompanied by the formation of intermediates, notably a disulfide between two CTX-A1 subunits. In the presence of PDI additional mixed disulfides of PDI with CTX-A2 and with CTX-A1 can be detected. Mixed disulfides with CTX-A1 could not only be detected in the test tube, but also in subcellular fractions isolated from CTX-treated Vero cells (Fig. 5).

CTX-A1 has to reach the cytoplasm in order to catalyze the NAD glycohydrolase and the ADP-ribosylation reactions. Clearly, the redox conditions prevailing in the cytoplasm (GSH/GSSG ratios between 20 and 80) would favor a rapid cleavage of CTX-A into CTX-A1 and CTX-A2. However, we do not know yet whether CTX-A or CTX-A1 (or both) is transported from the lumen of the ER to the cytoplasm. If the transport mechanism was specific for CTX-A1 the partial reduction of CTX-A in the ER would be a necessary step for the toxic action of CTX. For this step to occur it would also be important that CTX-A1 formed in the ER is only partially converted to protein mixed disulfides, as it seems unlikely that CTX-A1 is translocated to the cytoplasm as a protein-bound disulfide. Acid residues in the vicinity of a free cysteine inhibit the formation of mixed disulfides between ER proteins and unassembled immunoglobulin λ-chains [13]. In CTX-A1 an aspartate residue (Asp^{189}) is located close to the free cysteine (Cys^{187}). Whether this is sufficient to reduce the reactivity of Cys^{187} and thus protein-S-S-CTX-A1 formation remains an open question.

On the basis of our observations it seems possible that protein disulfides from other foreign or cellular proteins can be reduced in the ER.

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