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SOME PROPERTIES OF THE MEMBRANE-BOUND AND SOLUBILISED FORMS OF THE PROTEIN DISULPHIDE ISOMERASE OF RAT LIVER MICROSOMES

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Summary

1. An improved method of measurement of the protein disulphide isomerase (protein disulphide-isomerase, EC 5.3.4.1) activity of microsomal preparations is described.

2. This enzyme is shown to be released from the membranes into solution in the ultracentrifuge.

3. Some of the properties of the enzyme in the membrane bound and soluble forms are described.

4. The results lead to the suggestion that the enzyme present in rough membrane is different from the present in smooth membrane as well as being differently situated in the membrane.

5. Some of the effects of sucrose on the activity of the membrane bound enzyme are consistent with the view that the membranes may undergo irreversible conformational changes during preparation and storage.

Introduction

Enzyme catalysed thiol disulphide interchange has been postulated as a necessary stage in the biosyntheses of disulphide linked proteins [1], particularly the polymeric immunoglobulins [2]. An enzyme which catalyses the rearrangement of incorrect disulphide bridges in a variety of proteins has been indentified in the microsomal fractions of rat liver [3] and other mammalian tissue [4,5], in the microsomal fraction of baker's yeast and plant tissues [6]

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and in the cell wall membrane fraction of *Escherichia coli* after sonication [6]. The enzyme has been purified from bovine liver [7].

The various methods reported for assaying the activity of this enzyme have included the determination of the rate of regeneration of different proteins to their native forms starting with the reduced protein and assaying under mildly oxidising conditions [8] or starting with an incorrectly disulphide linked protein and assaying in the presence of a low concentration of mercaptoethanol [1]. The proteins used have been bovine pancreatic ribonuclease, hen egg-white lysozyme or soybean trypsin inhibitor [9]. A more direct method involved the determination of the rate of release of trichloroacetic-acid-soluble radioactivity from a mixed disulphide substrate prepared by the oxidation of reduced ribonuclease in the presence of [^{35}S]cysteine [10].

All of these methods suffer from one or a number of disadvantages. In several cases the poor storage properties of the substrates makes experimentation dubious and repeated preparations necessary. A high spontaneous rate of rearrangement of disulphide bonds in the substrate under the conditions of the assay but in the absence of the enzyme, seriously reduces the sensitivity of the measurement made. The presence of membrane material may interfere with spectrophotometric determinations of the activity of some of the regenerated proteins. Lysozyme dissolves in microsomal membranes (Roobol, A., unpublished).

The present study was undertaken to establish a more sensitive and reproduceable method of assay for the protein disulphide isomerase and to study some of its properties in the membrane bound state. In the course of this work it became apparent that the enzyme could be partially released from the membrane under the conditions pertaining during the normal procedure for preparation of rough and smooth microsomes. We have therefore re-examined the subcellular localisation of the enzyme and studied some of its properties in the soluble form.

Methods

Materials

Dithiothreitol and randomly reoxidised ribonuclease were purchased from Miles Laboratories Ltd.; the sodium salt of yeast RNA from Koch Light and bovine pancreatic ribonuclease A from Sigma (type XII-A). The buffers used were made up from 6.05 g Tris, 1.86 g KCl and 1.02 g $MgCl_2 \cdot 6H_2O$ per 1000 ml. Sucrose was added when required and the pH adjusted to 7.45 at 25° C with concentrated HCl before final dilution.

Preparation of Microsomes

Rough and smooth microsomes were prepared from 150 g male albino Wistar rats as described by Sunshine et al. and stored in aliquots as suspensions (10—20 mg membrane protein per ml in 0.25 M Sucrose, Tris/$MgCl_2$/KCl buffer at —32° C until required.

Reharvesting of membrane suspension (which had not been frozen) was performed by the addition of Tris/$MgCl_2$/KCl buffer (10 vol.) to the membrane suspended in 0.25 M Sucrose/Tris/$MgCl_2$/KCl buffer (1 vol. approx. 20 mg pro-

tein per ml) followed by centrifugation at 105 000 × *g* (average) in a "50Ti" angle rotor on a Beckman Spinco Model L ultracentrifuge for 60 min at 4° C.

Polyacrylamide gel electrophoresis

Membrane suspensions (approx. 5 mg protein per ml) were solubilised by the addition of a solution containing 2% sodium dodecyl sulphate, 10% sucrose and 4 M urea. Samples of supernatants from the reharvesting were densified with sucrose only. The separation of the proteins in these solutions on polyacrylamide gels was performed essentially as described by Hinman and Phillips [14].

Gels (75 × 5 mm) of 5%Cyanogum 41 (British Drug Houses) were polymerised in 1%dodecyl sulphate, 0.1 M Tris/acetate buffer (pH 9.0), 0.001%EDTA using $Na_2S_2O_8$ as polymerising agent. The gels wre pre-run at 1 mA per gel for 30 min. Samples of proteins were layered above the gels and electrophoresis carried out at 0.25 mA per gel for 30 min and then 1 mA per gel for 4.5 h. The gels were stained with Coomassie Brilliant Blue R.

Isoelectric Focusing in polyacrylamide gels

Samples of protein solutions were copolymerised with gels of 5% Cyanogum 41 in a 2% solution of Ampholines (pH range 3.5—10.0) containing 1 mM dithiothreitol and 4 M urea. Focusing was complete in 2 h. The Ampholines were washed from the gels with several changes of 12% trichloroacetic acid before staining with Coomassie Blue.

Protein, RNA and SH determinations

The concentrations of protein and RNA in microsome preparations were determined as described previously [6]. The reduction of bovine serum albumin [12] and the determination of thiol groups [13] were performed as described in the references cited.

Ultrafiltration

The Amicon Diaflo filter system was used for ultra filtration under the conditions specified by the manufacturer.

Determination of disulphide interchange activity

Disulphide interchange activity was determined by the enhancement of the rate of reactivation of randomly disulphide linked ribonuclease in the presence of dithiothreitol. Routinely an incubation at 30° C contained 0.8 ml Tris/$MgCl_2$/KCl buffer, 0.1 ml randomly refolded ribonuclease solution (0.5 mg per ml in 10 mM acetic acid), 50 µl dithiothreitol solution (0.1 mM in water) and 0.1 ml membrane suspension (2.5 mg protein per ml). Samples (10 µl) were taken for ribonuclease assay at time intervals from 2 to 32 min.

The 10-µl sample from the disulphide interchange incubation was added to a cuvetted (at 25° C) containing 3.0 ml of 0.25 M surcrose Tris/$MgCl_2$/KCl buffer and 50 µl RNA solution (5 mg per ml in water) in the primary cell compartment of a Perkin-Elmer 356 Spectrophotometer. The instrument was set to the "dual-wavelength" mode with 280 nm as reference wavelength and 260 nm as the measured wavelength. The initial slope of the plot of the output of the spectrophotometer, using the 0—0.1 absorbance scale, against time was

obtained on a recorder and taken as a measure of ribonuclease activity. A graph of ribonuclease activity against time was linear over the period of the disulphide interchange incubation. The slope of this graph was taken as a measure of disulphide interchange activity. Approximately 5—10% of the substrate was converted to native ribonuclease during the course of the assay. An arbitrary unit of disulphide interchange activity was used throughout the work which correponded to the generation of $1.6 \cdot 10^{-7}$ μmol of ribonuclease per min.

Results and Discussion

Measurement of ribonuclease activity

The initial slope of the recorder trace of the output from the spectrophotometer was found to be linearly related to ribonuclease concentration, under the conditions described, over the range required (0.1—1.2 mM). Variations of the conditions of the rearrangement incubation, described below, were found to cause only insignificant alterations of the conditions of the ribonuclease assay and did not change the ribonuclease activity measured.

The variation of disulphide interchange activity with mercaptan concentration

In the absence of added mercaptan no disulphide interchange activity was detected by this assay method in the presence of either rough or smooth microsomal membranes or the supernatants from reharvesting the membranes. Dithiothreitol stimulated maximum activity in rough and smooth microsomes and in the supernatant when present in the assay in concentrations between 2 and 10 μM (Fig. 1). At concentrations above or below this range the interchange activity measured was reduced. Mercaptoethanol and glutathione were also effective as membrane activators but maximum activity was only at much higher concentrations (0.5—1.0 mM). At these concentrations the stimulation of disulphide interchange by these mercaptans was considerable in the absence of microsomal material. The spontaneous rate of disulphide rearrangement was directly proportional to mercaptan concentration for all three mercaptans but was found to be considerably higher in the presence of 1 mM mercaptoethanol or glutathione than in the presence of 10 μM dithiothreitol. Concentrations of dithiothreitol above 50 μM completely inhibit the interchange reaction. Gurari [15] has reported that excess mercaptoethanol inhibits the enzyme. Only very slight activity (above the spontaneous rate) was observed in the presence of mercaptoethanol or glutathione for the supernatants from reharvesting the membranes.

A sample of reduced bovine serum albumin was prepared and found to catalyse the regeneration of ribonuclease in the assay system at the rate expected on the basis of its thiol content (Table I). Thus the possibility that a polythiol-containing protein might have an increased catalytic potency when compared with a simple mercaptan was excluded.

The thiol content of rough and smooth microsomes, determined by titration with 5,5′-dithio-bis(2-nitrobenzoic acid) was found to be less than 20 nmol -SH per mg protein.These observations are not consistent with the view that disulphide interchange is catalysed by microscomes because of endogenous

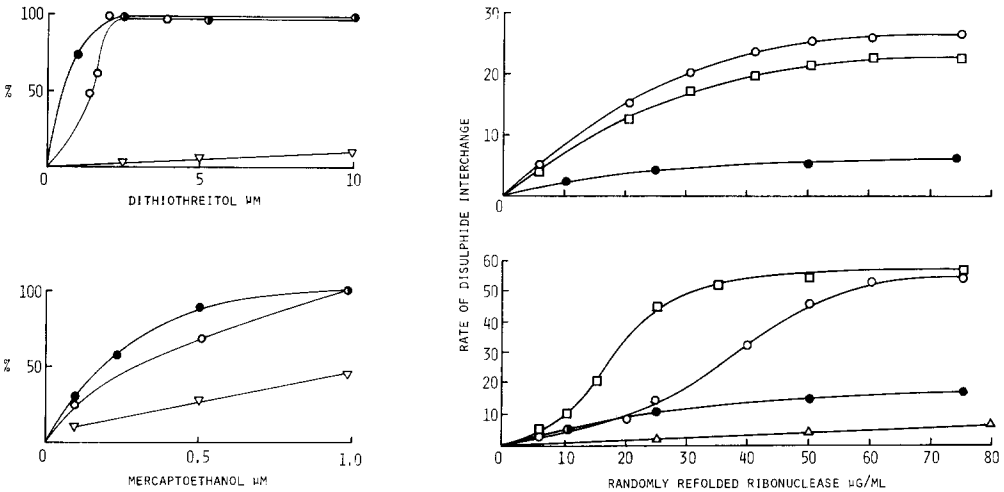


Fig. 1. Key: •, rough membrane; ○, smooth membrane; ▽, spontaneous. The effect of mercaptan concentration on the activity of the membrane bound protein disulphide isomerase. Membranes were assayed with different concentrations of dithiothreitol (above and mercaptoethanol (below) as described in methods. Activities (including spontaneous rates) are expressed as % of the activity of the membranes at 2.5 μM for dithiothreitol or 1.0 mM for mercaptoethanol.

Fig. 2. Key: □, Fresh membrane (assay buffer used Tris/KCl/$MgCl_2$): ○, Membrane stored for 48 h at 2°C in 0.625 M sucrose/Tris/KCl/$MgCl_2$. Assay buffer contained 0.0625 M sucrose: •, Fresh membrane. Assay buffer contained 0.83 M sucrose: △, Spontaneous rate in the absence of membrane. Variation with substrate concentration of the protein disulphide isomerase activity of rough and smooth membranes. The assay procedure and the definition of the unit of activity are given in Methods.

thiol containing proteins or incompleted proteins attached to the membrane. The thiol concentration of the membranes is insufficient to account for the activity observed and the need for an activating mercaptan is absolute. The observations are in accord with previous reports that partial reduction of randomly disulphide linked ribonuclease to give a substrate with approximately 3 -SH groups per molecule is sufficient to obviate the requirement of the enzyme for an activating mercaptan [1] but that no rearrangement occurs with the fully oxidised substrate unless a low concentration of mercaptan is present.

Effect of membrane protein concentration

The measured rate of disulphide interchange was found to be linearly proportional to membrane protein concentration for both rough and smooth microsomes over the range 0—0.5 mg protein per ml in the rearrangement incubation.

Variation of disulphide interchange activity with substrate concentration

The effect of variation in the substrate concentration on measured enzyme activity for rough and smooth membranes is shown in Fig. 2. Smooth microsomes show a velocity vs. substrate concentration curve which appears essentially hyperbolic. The rough membranes, however, show a markedly S-shaped curve. This curve may be altered by the sucrose concentration in the assay. There was found to be considerable inhibition by high sucrose concentrations

TABLE I

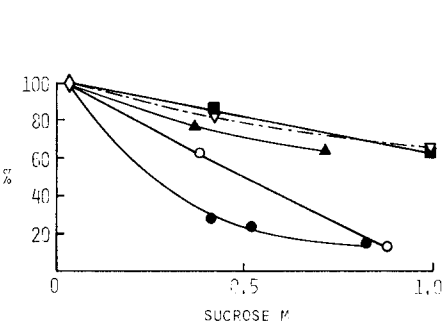
Sample	Specific activity (arbitrary units per mg protein) approx.	Approx. total activity recovered from 1 g wet weight of liver (arbitrary units)
10 000—300 000-dalton fraction of homogenate	less than 10	
10 000—300 000-dalton fraction of post-microsomal supernatant	40	95
Rough membrane (as normally prepared)	210	234
Smooth membrane (as normally prepared)	170	239
Rough membrane (after second harvest)	172	114
Smooth membrane (after second harvest)	250	157
Supernatant from second harvest of rough membrane	3900	223
Supernatant from second harvest of smooth membrane	1000	216
Reduced bovine serum albumin	11 *	—

* 1 mg per ml reduced bovine serum albumin corresponds to 0.51 mM -SH groups. 0.51 mM mercaptoethanol gives a rate of 12.5 units.

and, for rough membrane, an apparent loss of the S-shape. When assayed at high substrate concentration the sucrose inhibition is completely reversible for both membrane types. If rough membrane was incubated for 48 h in a high sucrose concentration the shape of the velocity vs. substrate curve was found to be altered but not the maximum rate obtainable. A similar effect was not observed for smooth membrane.

The effects of sucrose are unexpected. It has been reported that high sucrose concentrations assist the preservation of the protein synthesising capacity of rough membrane [16]. We have found that the disulphide interchange enzyme activity is better preserved for both membrane types at 0—4°C in Tris/$MgCl_2$/KCl buffers containing high sucrose concentrations (more than 0.6 M) than in buffers containing 0.25 M sucrose or less. The change in the shape of the velocity vs. substrate curve for rough membrane on incubation in high sucrose buffer (which cannot be accounted for simply on the basis of the slight change in sucrose concentration in the assay medium) is difficult to explain except in terms of some slowly reversible or irreversible change in membrane structure. The possibility that such conformational changes occur during the course of normal preparation and storage of microsomes cannot be excluded. The inhibition by sucrose of the activity of the soluble enzyme preparations (Fig. 3) is much less marked than for the membranes.

It should be noted that such S-shaped velocity vs. substrate curves can be



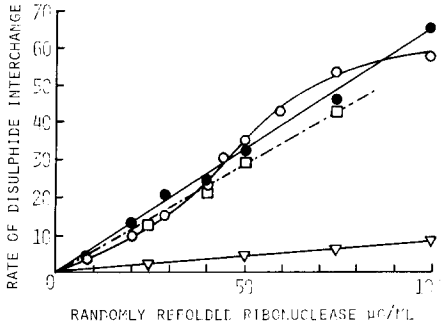


Fig. 3. Key: ●, rough membrane; ○, smooth membrane; ■, supernatant from reharvesting rough membrane; ▲, supernatant from reharvesting smooth membrane; ▽, bovine enzyme sample. Variation with sucrose concentration in the assay of the protein disulphide isomerase activity of membranes, supernatants from reharvesting the membranes and the partially purified bovine enzyme. The assay procedure is described in Methods. The activities are expressed as % of that obtained when the assay contained 0.025 M sucrose.

Fig. 4. Key: ●, supernatant from reharvesting smooth membrane; ○, supernatant from reharvesting rough membrane; □, bovine enzyme sample; ▽, spontaneous rate (in the absence of enzyme). Variation with substrate concentration of the protein disulphide isomerase activity of the soluble enzyme preparations. Assays of the supernatants contained 20 μg per ml protein. The bovine enzyme was assayed using 50 μg per ml.

observed under certain conditions for enzymes which do not show co-operativity in substrate binding [17,18]. The possibility of diffusion limited reaction rates [18] seems particularly plausible in the case of a membrane bound enzyme acting on a macromolecular substrate.

The determination of the variation of the rate of the disulphide interchange reaction with substrate concentration for the supernatants from reharvesting rough and smooth membranes (Fig. 4) showed some differences from the membrane bound enzymes. No saturation was observed with substate concentrations up to 100 μg per ml for the enzyme derived from smooth membrane. Similar behaviour was observed for the bovine enzyme partially purified by the method of Lorenzo et al. [7]. The enzyme derived from rough membrane showed saturation within this substrate concentration range and a markedly S-shaped curve. This observation must lead to the suggestion that the enzyme present in rough membrane is in some way structurally different from that present in smooth as well as being differently located in the membrane [6].

The effects of temperature on the interchange reaction

The variation of the measured interchange activity with temperature for rough and smooth microsomes was determined. An optimum temperature for the activity of rough membrane was found around 32°C whereas in smooth membranes a broad plateau of maximal activity was observed between 30 and 40°C. It is noteworthy that the progress curves for the interchange reaction were observed to be linear for at least 30 min over the temperature range studied suggesting that the fall-off in enzyme activity at high temperature is not due to denaturation of the enzyme but to some other factor possibly membrane structural changes. The activities of several microsomal enzymes have been shown to undergo abrupt changes near 32°C [19].

The effects of pH on the interchange reaction

The variation with the pH of the assay medium of the interchange activity of rough and smooth membranes and of the supernatants from reharvesting the rough and smooth membranes was found to be complex. The experiments covered the range of pH practicable with this buffer system (pH 7.0 t0 9.0). Rough membranes appear to show 2 superimposed pH profiles, one with an optimum around pH 7.5 and another with an optimum above pH 8.4. The smooth membranes show similar but not identical behaviour. Both rough and smooth membranes showed similar biphasic behaviour when mercaptoethanol (1 mM) replaced dithiothreitol in the assay medium. Membranes which had been harvested a second time were found to exhibit a simpler pH profile without the optimum at pH 7.5. The enzymes released into the supernatant during the reharvesting procedure showed no optima within this pH range.

Biphasic pH curves have been observed for enzymes on membrane-like supports [20] and the activation of several microsomal membrane bound enzymes by treatment of the membranes at high pH has been reported [21,22]. It has been suggested that this effect is associated with irreversible changes in the membrane structure rather than in the enzymes themselves [22]. It may reasonably be presumed that the change in pH profile of the membranes after reharvesting also reflects an effect on membrane structure.

Subcellular localisation of the interchange enzyme

The occurrence of certain enzymes in the microsomal fraction of liver homogenates, particularly fumarase [23] aldolase and glutamine synthetase [21] has been attritubed, at least in part, to a redistribution of the enzymes during the homogenisation and subsequent fractionation procedures. It has also been reported that the allosterically controlled nuceoside diphosphatase [25], a typical microsomal enzyme [24], may be released from the membranes under the conditions usually existing in the ultracentrifuge during the preparation of rough and smooth microsomes [26]. In view of the distribution of enzyme activity observed during fractionation, shown in Table I, we have re-examined the subcellular localisation of the protein disulphide isomerase in order to determine whether one of the above phenomena account for this distribution.

The 10 000—300 000-dalton fraction was separated by ultrafiltration from a diluted liver homogenate and from the post microsomal supernatant after ultracentrifugation. Enzyme activity was detected in the latter but not the former (Table I). This experiment is consistent with the subcellular localisation previously reported [8] for this enzyme and indicate that the occurrence of interchange activity in the supernatant from reharvesting the membranes and in the post microsomal supernatant is principally due to the release of the enzyme from the membranes and not to the existence of the enzyme in the cytoplasm in vivo.

The possibility that the enzyme activity present in the supernatants from reharvesting the membranes represents unsedimented membrane fragments was excluded since the activity was associated with proteins of mol. wt. not greater than 300 000. The considerable increase in specific activity in the supernatants compared with the membranes (Table I) and the much simpler protein mixture (Fig. 5) existing in the supernatants are additional confirmation that

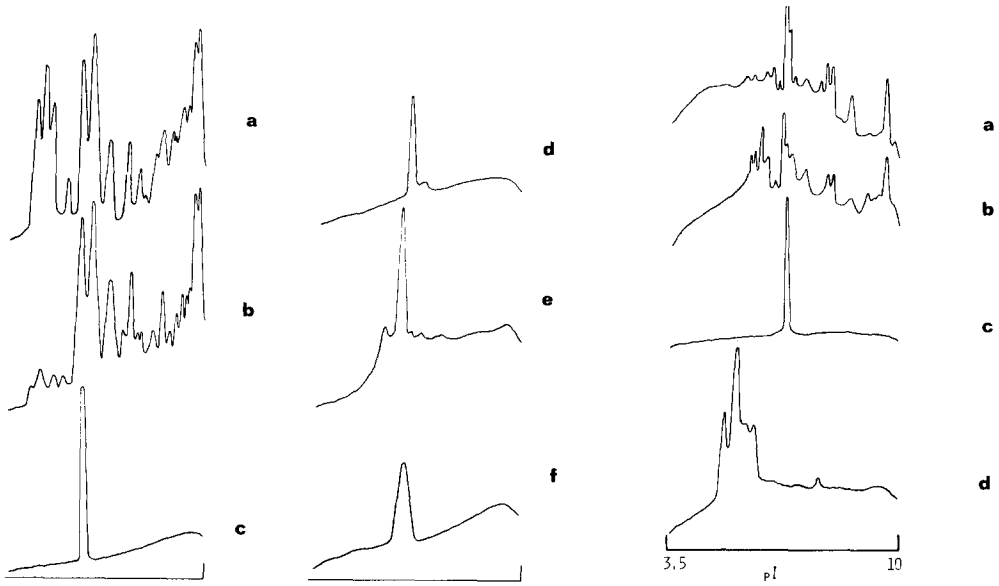


Fig. 5. Sodium dodecyl sulphate polyacrylamide gel electrophoresis of membranes and supernatants from reharvesting the membranes. Gels were run as described in Methods, the proteins move towards the anode (left hand side of trace) 30 μg protein applied to gels for membrane samples and 10 μg for soluble protein samples. After staining the gels were scanned using a Joyce-Loebel ultraviolet scanner. (a) Rough membrane. (b) Smooth membrane. (c) Rat serum albumin. (d) Supernatant from second harvest of rough membrane. (e) Supernatant from second harvest of smooth membrane. (f) Partially purified bovine liver protein disulphide isomerase.

Fig. 6. Isoelectric focussing of soluble proteins. 10—15-μg protein samples applied and electrofocussing was performed as described in methods. After staining the gels were scanned using a Joyce-Loebel ultraviolet scanner. (a) Supernatant from second harvest of rough membrane. (b) Supernatant from second harvest of smooth membrane. (c) Rat serum albumin. (d) Partially purified bovine liver protein disulphide isomerase.

the enzyme is really soluble. The analysis of the proteins present in the supernatants (Figs. 5 and 6) indicate that one major component has a mol. wt. and isoelectric point similar to serum albumin. A number of other components are present, however, the adequate resolution of which was found to be difficult even in the presence of disaggregating agents. The complete purification of this enzyme is consequently likely to be extremely difficult.

In view of the differences reported in the rate of turnover of different microsomal proteins [27] and the possibility that proteins may leave the membrane and enter the cytoplasm of the cell before catabolism [27], the release of membrane components may be considered a normal physiological process the mechanism and control of which may merit further study.

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