Protein disulphide-isomerase activity in chick-embryo tissues

Correlation with the biosynthesis of procollagen

Barbara E. BROCKWAY, Simon J. FORSTER and Robert B. FREEDMAN
Biological Laboratory, University of Kent, Canterbury CT2 7NJ, Kent, U.K.

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Protein disulphide-isomerase activity was determined in homogenates of chick-embryo tissues. Activities were highest in tissues active in procollagen synthesis and were maximal at the developmental stage of maximal procollagen synthesis. These variations in protein disulphide-isomerase activity correlate closely with those previously observed for procollagen hydroxylase activities.

The mechanism by which disulphide bonds are formed during the biosynthesis of proteins is not known; neither the immediate oxidant nor the catalyst have been firmly identified (Freedman & Hawkins, 1977; Ziegler & Poulsen, 1977). Studies *in vitro* on the formation of disulphide bonds in reoxidation of reduced proteins indicate that the rate-determining steps are conformational changes accompanied by intramolecular thiol–disulphide interchange reactions in which non-native disulphide pairings are replaced by native disulphide pairings (Creighton, 1978). An enzyme has been isolated that catalyses such reactions. This enzyme, protein disulphide-isomerase (EC 5.3.4.1), is widely distributed in animal and plant tissues, is found in the microsomal fraction in mammalian liver, has a wide specificity for protein disulphide substrates but little activity towards low-molecular-weight disulphides [see Freedman & Hillson (1980) for a review]. The enzyme has been purified from bovine liver (Hillson & Freedman, 1980), and its action during formation of native disulphide bonds *in vitro* has been clarified (Creighton *et al.*, 1980).

It has been difficult to establish definitely whether this enzyme is involved in the biosynthesis of protein disulphide bonds, mainly because of the large number of cellular processes involving interchange between thiols and protein disulphides, and the difficulty in assigning a physiological role to an enzyme of broad specificity (Freedman, 1979). To date the enzyme has been mainly studied in liver, and it is possible that more clear-cut indications of the enzyme's physiological role would come from studies on cells that are less versatile metabolically and synthesize a single major disulphide-linked protein.

In view of this, we have begun a study of protein disulphide-isomerase in tissues active in the syn-

thesis, assembly and secretion of procollagen, such as chick-embryo tendon, cartilage and bone. All procollagens contain interchain disulphide bonds, which link the C-terminal globular domains of procollagen polypeptides. The formation of these interchain disulphide bonds is important in the folding and assembly of procollagen. There is a close correlation between the timing of disulphide formation and the formation of the native triple-helical conformation; disulphide-bonding is a necessary, but not sufficient, condition for folding into the triple helix [see reviews by Bornstein (1974), Grant & Jackson (1976) and Fessler & Fessler (1978)]. Studies on the refolding of procollagen fragments and model-building have suggested that a 'knot' of disulphide bonds between three polypeptide chains brings the chains into a conformation in which the triple-helical structure can be rapidly propagated along the chains in the C-terminal-to-N-terminal direction (Bruckner *et al.*, 1978; Bächinger *et al.*, 1978).

In preliminary work we have shown that protein disulphide-isomerase is present with significant activity in chick-embryo tendon and cartilage microsomal fractions (Harwood & Freedman, 1978). Procollagen is the predominant protein synthesized by these tissues. They therefore offer an ideal system in which to test the hypothesis that protein disulphide-isomerase is involved in protein disulphide formation *in vivo*, by examining whether the enzyme is present in the appropriate cells during the appropriate developmental stage and at the appropriate subcellular location to make it plausible that it acts on newly synthesized procollagen. Our results show that the activity of protein disulphide-isomerase in chick-embryo tissues is closely correlated with procollagen-synthesizing activity and in particular that the variation in isomerase

activity between tissues and as a function of embryonic development is parallel to the variation in the activity of lysine and proline hydroxylases.

Experimental

Fertile eggs, obtained from Golden Produce (Hatchery and Farms Division), Lydd, Kent, U.K. were incubated at 37°C in a moist atmosphere in a Westernette egg incubator (Western Incubators, Burnham-on-Crouch, Essex, U.K.). The following tissues were dissected out, pooled, rinsed and homogenized; tendon (from the foot), cartilage (from the sternum), leg bones (tibia in the case of embryos from 15 days onwards, total leg bones in the case of younger embryos), frontal bones of the cranium, skin (from the breast), muscle (from the leg), heart, liver and brain. Pooled tissues from between 20 and 100 embryos were rinsed in a large volume of S-TKM buffer (0.25 M-sucrose/50 mM-Tris/25 mM-KCl/5 mM-MgCl₂ adjusted to pH 7.5 with HCl) and then homogenized in 2 vol. of this buffer. Brain, liver and heart were homogenized by using 20 strokes of a motor-driven Teflon/glass homogenizer with a 0.1 mm gap. The remaining tissues were first homogenized by using the Ultra-Turrax Blender at maximum speed, ten 15 s bursts with 1 min intervals between bursts being used; the resultant material was then further homogenized with the Teflon/glass homogenizer as described for the soft tissues. All these operations were carried out on

ice. The crude homogenates were centrifuged at 600 *g*_{av} for 5 min to sediment debris, and the supernatants were then assayed for protein disulphide-isomerase activity. The assay, which is based on the appearance of ribonuclease activity in inactive, 'randomly' disulphide-linked ribonuclease is described in full by Ibbetson & Freedman (1976), where the units of protein disulphide-isomerase activity are also defined. Protein was determined by the method of Lowry *et al.* (1951).

Randomly reoxidized ribonuclease was obtained from Miles Laboratories, Stoke Poges, Slough, Berks., U.K.; high-molecular-weight yeast RNA was from Koch-Light, Colnbrook, Bucks., U.K.; Tris and dithiothreitol were from Sigma, Poole, Dorset, U.K. All other reagents were from Fisons, Loughborough, Leics., U.K., and were AnalaR grade where possible.

Results and discussion

The distribution of protein disulphide-isomerase activity between chick-embryo tissues was studied in homogenates from 17-day embryos. Activity was reproducibly found in the order: tendon > cartilage > tibia ≈ cranium > skin ≈ muscle ≈ heart > liver > brain. Thus activity is highest in connective tissues, and the distribution shows a clear correlation with the rate at which tissues synthesize extracellular matrix proteins such as collagen. Table 1 shows a comparison between the distribution

Table 1. *Protein disulphide-isomerase activity of chick-embryo tissues*

Tissue samples from between 20 and 100 17-day chick embryos were pooled, and homogenates were prepared as described in the Experimental section. Assays of protein disulphide-isomerase (Ibbetson & Freedman, 1976) and protein (Lowry *et al.*, 1951) were performed in duplicate or triplicate. The data presented are mean activities from two separate experiments in which all nine tissues were dissected, homogenized and assayed in parallel from single batches of embryos. All tissues were assayed on other occasions by using various batches of embryos. Means [$\pm$ s.d.(*n*)] of all the data obtained are: tendon 1.60 ± 0.66 (6), cartilage 1.21 ± 0.68 (3), tibia 0.64 ± 0.02 (4), cranium 0.94 ± 0.53 (3), skin 0.35 ± 0.15 (3), muscle 0.30 ± 0.16 (3), heart 0.35 ± 0.18 (3), liver 0.20 ± 0.05 (5) and brain 0.08 ± 0.05 (3).

Tissue	Enzyme activity			
	Protein disulphide-isomerase		Proline 4-hydroxylase	Lysine-hydroxylase
	(units/g)	(%)	(%)*	(%)†
Tendon	1.97	100	100	100
Cartilage	1.44	73	—	—
Tibia	0.80	41	55	69
Cranium	0.65	33	31	48
Skin	0.44	22	24	27
Muscle	0.39	20	—	—
Heart	0.36	19	14	9
Liver	0.22	11	6	5
Brain	0.11	6	—	—

* Data from Tryggvason *et al.* (1979).

† Data from Ryhanen & Kivirikko (1974). Similar methods of sample preparation were used in these studies and in ours. Lysine hydroxylase activities were determined as activity/wet weight of tissue, whereas, others were determined as activity/mg of protein.

observed for protein disulphide-isomerase and those found by others for the three procollagen hydroxylases; these enzymes act almost exclusively on nascent or unfolded procollagen and have been proposed as quantitative markers for procollagen biosynthesis [see Kivirikko & Myllylä (1980) for a review]. All four enzymes show parallel tissue distributions (the orders of activity are identical, except that heart > skin for proline 3-hydroxylase), and there is surprisingly close quantitative agreement between the distribution of protein disulphide-isomerase, proline 4-hydroxylase and lysine hydroxylase. Differences between the relative activities of isomerase and the hydroxylases are no greater than those between the hydroxylases themselves.

The implication from these data that protein disulphide-isomerase may be involved in post-translational modification of procollagen is strengthened by studies in which isomerase activity was monitored in chick-embryo leg-bone and liver homogenates as a function of embryonic development. As shown in Fig. 1, isomerase specific activity in bone increased to a maximum at day 15 and then declined markedly up to day 19, whereas activity in liver showed no significant variation over this period. The time course of procollagen synthesis in chick embryo is well-established; the protein can be detected at very early stages of development and its specific rate of synthesis increases as development proceeds, reaching a maximum in whole embryo and in particular tissues between day 14 and day 16 of development and decreasing thereafter (Diegelmann & Peterkovsky, 1972; Ryhanen & Kivirikko, 1974). The changes in specific rate of synthesis of procollagen are reflected in changes of specific activity of the procollagen hydroxylases (Halme, 1969; Ryhanen & Kivirikko, 1974; Tuderman, 1976). Data on developmental changes in the hydroxylases are also plotted in Fig. 1 for comparison with the changes in protein disulphide-isomerase. For all three enzymes it is apparent that the activity in bone increases to day 14–15 and then decreases, that the activity in liver is constant over the period 10–19 days, and that the activity in liver is 10–30% of that in bone (cf. Table 1). This closely similar pattern of developmental changes between protein disulphide-isomerase and the procollagen hydroxylases suggests that they are all involved in the same cellular process, namely the post-translational modification of procollagen.

In view of the importance of interchain-disulphide-bond formation to the folding and assembly of procollagen and to the control of other post-translational modifications of the protein (Grant & Jackson, 1976), it is likely that this disulphide-bond formation would be enzymically catalysed. The results above show that tissues in which pro-

Fig. 1. Variation of protein disulphide-isomerase activity as a function of embryonic development

Isomerase activity was assayed in homogenates of bone (Δ) and liver ($\blacktriangle$) pooled from 20–100 embryos. Points shown are means for three independent preparations (13 days) or of two independent preparations (11, 15 and 17 days); single preparations only were made from embryos at day 12 and day 19. The data are expressed as percentages of the maximum activity attained for the enzyme in bone tissue, namely $0.74 (\pm 0.02)$ unit/g. Mean embryo weights were approx. 3, 9, 17, 26 and 34 g on days 11, 13, 15, 17 and 19 respectively. Data on proline hydroxylase ($\square, \blacksquare$) from Halme (1969) and on lysine hydroxylase ($\circ, \bullet$) from Ryhanen & Kivirikko (1974) are also shown. Open symbols refer to bone homogenates, closed symbols to liver homogenates. Data are expressed as percentages of the maximum activity attained for each enzyme in bone homogenates.

collagen is the major product of protein synthesis contain protein disulphide-isomerase, an enzyme that catalyses disulphide formation and interchange in a wide range of proteins and that has been proposed to be involved in disulphide formation in protein biosynthesis. The tissue distribution of chick-embryo protein disulphide-isomerase and its variation with development, together with our earlier detection of the enzyme in microsomal fractions from chick-embryo tendon and cartilage (Harwood & Freedman, 1978), indicate that this enzyme is

located in the appropriate cells at the appropriate subcellular location and at the appropriate developmental stage to play a part in the assembly of procollagen. This is the first report of a clear correlation between the presence of protein disulphide-isomerase and the biosynthesis of a specific disulphide-bonded protein.

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