containing fused α-subunits is increased 3-4-fold compared with its non-fused homologue. We also studied the effects of rHb1.1 on renal function in dogs, removing 30% of the blood volume and replacing it with an equivalent volume of saline, 50 mg ml^{-1} human serum albumin, 50 mg ml^{-1} rHb1.1, or with the animal's own blood. Serum creatinine, blood urea nitrogen, creatinine clearance rate and excretion of *N*-acetyl-β-D-glucosaminidase were measured as indicators of glomerular function: serum creatinine and urea nitrogen increase and creatinine clearance is slower if glomerular function is compromised. Increased *N*-acetyl-β-D-glucosaminidase in the urine is a sensitive measure of papillary damage in the kidney of dogs[20]. The results in Table 2 show that kidney function was normal in all groups. Kidney histology was also normal in all groups at 7 or 14 days after administration of the test solution (data not shown), and no haemoglobin was detected in the urine of the animals (limit of detection is 1.5 μg ml^{-1}; data not shown). These results are in contrast to the dramatic nephrotoxicity of unmodified haemoglobin[8,9,21].

Unlike haemoglobins that have been chemically crosslinked, genetically fused di-α-haemoglobin can be produced in large amounts by simple microbial fermentation and purified without further modification. Our engineered haemoglobin is therefore a strong candidate for a safe blood substitute. □

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Role of ATP and disulphide bonds during protein folding in the endoplasmic reticulum

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BEING topologically equivalent to the extracellular space, the lumen of the endoplasmic reticulum (ER) provides a unique folding environment for newly synthesized proteins. Unlike other compartments in the cell where folding occurs, the ER is oxidizing and therefore can promote the formation of disulphide bonds[1]. The reducing agent dithiothreitol, when added to living cells, inhibits disulphide formation with profound effects on folding[2]. Taking advantage of this effect, we demonstrate here that folding of influenza haemagglutinin is energy dependent. Metabolic energy is required to support the correct folding and disulphide bond formation in this well characterized viral glycoprotein, to rescue misfolded proteins from disulphide-linked aggregates, and to maintain the oxidized protein in its folded and oligomerization-competent state.

To monitor the folding process, we used a previously developed pulse-chase assay in influenza-infected cells[3]. At the end of each chase time, the cells were treated with a membrane permeant alkylating agent, *N*-ethyl maleimide (NEM), to trap folding intermediates[4]. The formation of disulphide bonds was followed by the changing mobility of haemagglutinin (HA0) bands in nonreducing SDS-PAGE. Conformational comparisons were made by immunoprecipitation with a panel of monoclonal and polyclonal antibodies.

The post-translational folding and maturation of HA0 in unperturbed CHO15B cells is shown in Fig. 1. After a 2-min pulse, two major folding intermediates, IT1 and IT2, were visible as well as the fully oxidized, untrimmed HA0 (called NT)[3]. During the chase, IT1 and IT2 were converted to NT, which then trimerized (not shown) and moved to the Golgi complex where its carbohydrates were trimmed, resulting in a faster moving band marked G (for Golgi) (Fig. 1, lanes 4-6). When reduced, IT1, IT2, and NT migrated as a single band (R) with a mobility slightly lower than IT1. The trimmed G (GR) was resolved again as a faster moving band (lane 7). When dithiothreitol (DTT) was added to the chase medium, the radioactively labelled IT1, IT2 and NT were reduced. The reduced form R appeared whether the cells were exposed to ATP depletion or

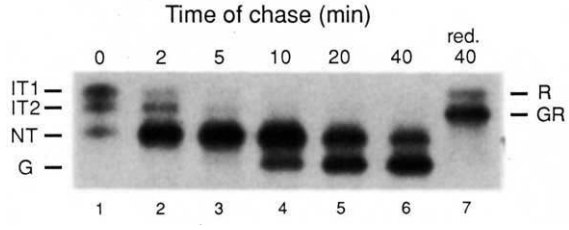

FIG. 1 Post-translational folding of HA0 in CHO15B cells. Two folding intermediates with an immature set of disulphide bonds (IT1 and IT2) gradually folded into the native form (NT), which has all 6 disulphide bridges. Transport out of the ER is apparent from the increase in mobility (G), caused by trimming of carbohydrates from $Man_9GlcNAc_2Asn$ to $Man_5GlcNAc_2Asn$. When reduced, IT1, IT2 and NT ran as one band (R) (lane 7). GR denotes the reduced Golgi-form of HA0. The CHO15B cells were used in our experiments because they are defective in *N*-acetylglucosaminyltransferase I[29,30], and the trimmed $Man_5GlcNAc_2Asn$ form of the *N*-linked carbohydrates cannot be processed further. This allows easy distinction between pre-Golgi (NT) and later forms (G) of HA0 by SDS-PAGE.
METHODS. Folding of HA0 was monitored as described before[3]. Briefly, 6 h after infection with X31 influenza virus (derived from A/Aichi/1968/H3N2), CHO15B cells were pulse-labelled for 1 min with 125 μCi ml^{-1} each of [^{35}S]cysteine and Tran[^{35}S]label. A chase with 5 mM methionine and 5 mM cysteine followed, with 0.5 mM cycloheximide in the medium to block elongation of labelled nascent chains. Ice-cold PBS with 20 mM *N*-ethylmaleimide stopped the chase and prevented rearrangement of disulphide bonds. This concentration of *N*-ethylmaleimide was at least 10-fold the concentration needed to consistently preserve IT1 and IT2. The cells were lysed in ice-cold 0.5% Triton X-100 in MNT (20 mM MES, 100 mM NaCl, 30 mM Tris-HCl, pH 7.5) containing 20 mM *N*-ethylmaleimide, 1 mM EDTA, 1 mM PMSF and 10 μg ml^{-1} each of chymostatin, leupeptin, antipain and pepstatin. Lysates were immunoprecipitated with a polyclonal rabbit antiserum against X31 virus (P), which recognizes all forms of the haemagglutinin[3,7,8]. Reducing and nonreducing SDS-PAGE (7.5%), followed by fluorography, was used to analyse the folding process.

not (Fig. 2, lanes 2 and 5). The ATP-depleting medium used was glucose-free and contained sodium azide and 2-deoxy-D-glucose[5,6]. When the DTT was washed out, rapid reoxidation occurred. In control cells, IT1 and IT2 reappeared, NT was formed (lanes 3, 4), and the protein was transported to the Golgi complex.

In energy-depleted cells, IT1, IT2 and NT were only seen transiently (lanes 6, 7) before HA0 moved into aggregates on top of the gel. The aggregates increased in size with time, resulting in material that would not enter the stacking gel. If the samples were reduced before electrophoresis, the labelled HA0 in the aggregates was quantitatively recovered as a reduced, nontrimmed monomer (R), indicating that the HA0 in the energy-depleted cells was not being degraded, that it was confined to the ER, and that it was present in disulphide crosslinked complexes. When DTT was already present during translation, HA0 was synthesized in a completely reduced form (R; Fig. 2, lane 10). After DTT wash-out under energy-depleting conditions (Fig. 2, lanes 12–15), it also aggregated, but without the transient formation of IT1, IT2 or NT, indicating that the fully reduced HA0 could not fold properly in energy-depleted cells.

To determine whether folding during normal HA0 biosynthesis also required metabolic energy, we pulse-labelled cells and then depleted them of ATP during the chase. DTT was not added at any stage of the experiment. Again, disulphide-linked

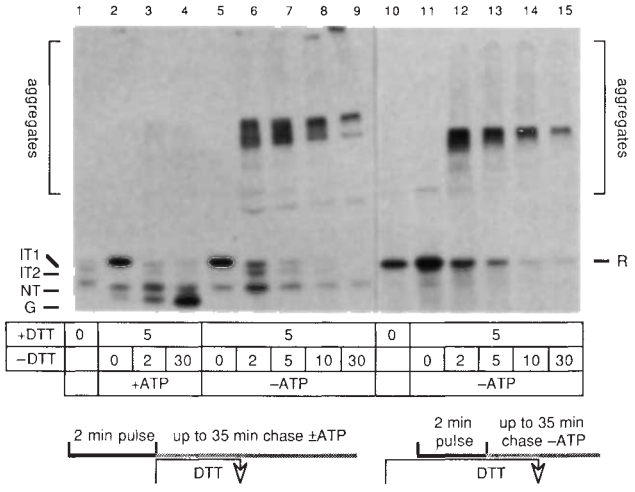

FIG. 2 ATP dependence of disulphide-bond formation. CHO15B cells were pulse-labelled for 2 min. DTT (5 mM) was then added in the chase. Post-translational reduction of HA0 was very fast *in vivo* (lane 2). When DTT was washed out in the presence of ATP, only a very small part of the protein was trapped into an aggregate by aberrant interchain disulphide bond formation (lane 3). These aggregates were dimers of HA0, trimers, and larger clusters, and they did not change over time (lane 4). Reoxidation in the absence of ATP, however, landed the major part of HA0 in disulphide-linked aggregates (lanes 6–9), which gradually increased in size. When reduced before SDS–PAGE, HA0 ran as one band (not shown). None of the HA0 was allowed to exit the ER. Lanes 10, 11 show HA0 synthesized in the presence of 5 mM DTT. When DTT was now washed out in the absence of ATP (lanes 12–15), the same aggregates appeared as in lanes 6–9, also increasing in size. In contrast to this, however, no oxidized forms of HA0 appeared at any time.

METHODS. Procedures were essentially as described in Fig. 1. Lanes 1–9, 2-min pulse followed by a chase with 5 mM DTT to reduce HA0 *in vivo*. The reducing agent was then removed by two changes of chase medium without DTT followed by an incubation of up to 30 min. In lanes 10–15, the DTT was also added 5 min before and during the pulse. Care was taken that all cells received the same concentration of DTT for the same time before the pulse started. In lanes 5–15, the cells were depleted of ATP during the chases with and without DTT. The ATP-depleting system consisted of glucose-free DMEM with 20 mM 2-deoxy-D-glucose and 10 mM sodium azide.

TABLE 1 Conformation of various forms of HA0 determined by immunoprecipitation

	F1	A1	anti-BiP
HA0 intermediates in the unperturbed cell			
IT1	+++	−	−
IT2	−	−	−
NT	−	−	−
G	−	−	−
HA0 after translation in the presence of DTT			
R (no chase)	+++	−	−
R (5′ chase + DTT − ATP)	+++	+++	+
HA0 aggregates after wash-out of DTT in the absence of ATP			
after translation +DTT	+++	+++	+
after post-translational reduction	+++	+++	+

Detergent lysates containing the different forms of radioactively labelled HA0 were used for immunoprecipitation with the conformation-specific antibodies F1 and A1 against HA0 and with the monoclonal anti-BiP antibody. Immunoprecipitations were done on detergent lysates (Fig. 1) as described[3]. The antibodies used were from the following sources: F1 is a mouse monoclonal IgG made against nondenatured HA2 (ref. 3), and A1 is a mouse monoclonal antibody made against acid-treated HA0, which recognizes misfolded forms of HA0[7,8]. The monoclonal antibody to BiP precipitates native BiP[10] as well as complexes between BiP and misfolded HA0[7,10–13,28]. The fraction of HA0 recognized by the antibodies was roughly estimated and compared with the amount immunoprecipitated by the polyclonal antiserum P (=100%): +, <40%; +++, >80%. IT1, IT2, NT and G are defined as in Fig. 1, and R stands for any reduced form of HA0.

aggregates were formed (Fig. 3*a*, lanes 2–5). IT1 and IT2, as well as NT formed during the pulse, disappeared and moved into the covalently linked aggregates, indicating that already folded proteins were modified and trapped into aggregates when the energy level dropped. We also tested what would happen if cells were allowed to regenerate ATP after the formation of aggregates. The results in Fig. 3*a*, *b* (lanes 6, 7 and 5, 6, respectively) showed that a large fraction of the labelled HA0 returned to a monomeric form and proceeded to fold normally. The misfolded molecules in the disulphide crosslinked aggregates were thus rescued when metabolic energy was made available again.

The aggregates observed in the various energy-depleted cells represented misfolded HA0. In addition to having aberrant interchain disulphide bonds, they were precipitated by monoclonal antibody A1 which is specific for misfolded and acid-treated HA0[7,8], and by antibody F1 which sees the first folding intermediate IT1 but not IT2, normally folded monomers (NT) or mature HA0 trimers (Table 1). The major top domain epitopes A, B and E, present in all normal forms of HA0, were only marginally expressed (not shown). Moreover, the aggregates coprecipitated with BiP (or grp 78)[9,10], a property of a variety of misfolded forms of HA0[7] and other proteins[11–13]. Although the anti-BiP antibody precipitates folding intermediates of numerous other proteins, it did not precipitate any of the normal maturation intermediates of X31 HA0 (Table 1). The antibody precipitation experiments indicated that misfolding also occurred when ATP was depleted in the presence of DTT (Table 1, line 6), that is under conditions where no disulphides could form. The misfolded conformation after depletion of metabolic energy was, therefore, not directly dependent on either the presence of aberrant or the absence of native disulphide bonds.

Our results revealed that metabolic energy was required to secure the correct folding of newly synthesized HA0, to rescue misfolded HA0 molecules from disulphide crosslinked aggregates, and to maintain already folded monomeric HA0 in a folded state. They suggested that HA0 in the ER is subject to

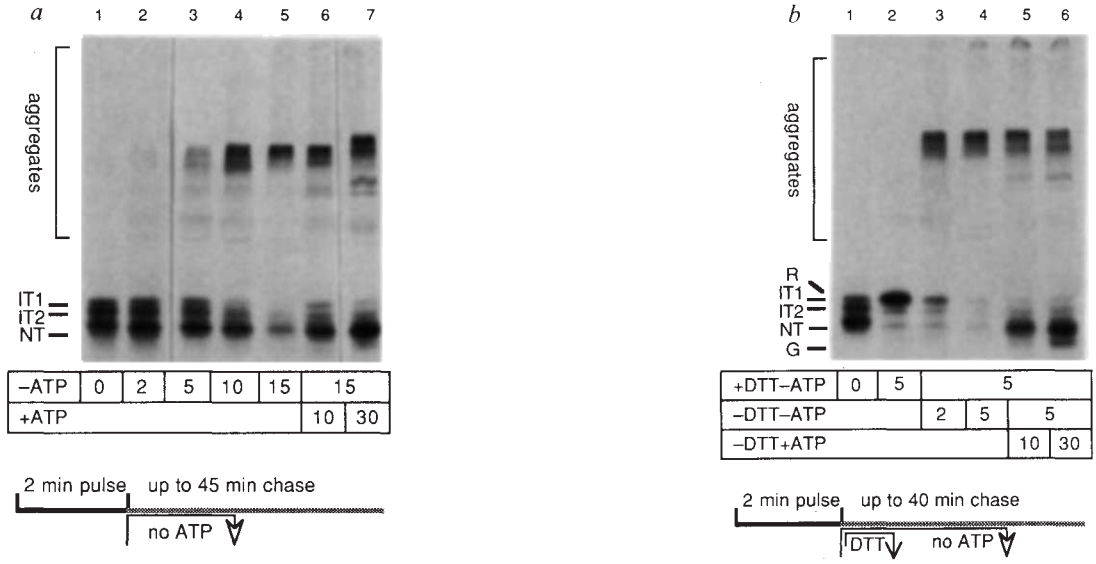

FIG. 3 ATP dependence of HA0 folding and refolding. *a*, When ATP was depleted during a chase immediately after pulse-labelling, HA0 formed disulphide-linked aggregates, which increased in size during the chase. The slow onset of this aggregate formation was most probably caused by the delay in the ATP depletion. The aggregates were similar to the ones observed after previous reduction of HA0 (Fig. 2, lanes 6–9). When cells were allowed to recover from the ATP depletion, a large fraction of the HA0 returned from the aggregates into folding intermediates, folded into NT (lanes 6–7), trimerized, and was transported to the Golgi. *b*, After ATP depletion during post-translational reduction and reoxidation, cells were allowed to recover (lanes 5, 6). Aggregates decreased in size, and HA0 reappeared on the gel as IT1, IT2 and NT. After a longer recovery time, the correctly folded NT was trimerized and transported, as judged by the presence of G (lane 6).
METHODS. *a*, Conditions were as in Fig. 1, with one exception: the chase medium consisted of glucose-free DMEM with 20 mM 2-deoxy-D-glucose and 10 mM sodium azide to deplete ATP in the cells. After 15 min, this medium was replaced with regular medium to allow recovery. *b*, Conditions were as in Fig. 2, lanes 5–9. After 10 min total chase, the ATP-depleting medium was replaced by regular chase medium by two washes, to allow the cells to recover from the ATP depletion.

continuous folding, unfolding and refolding with energy required for folding and refolding. Because HA0 monomers (NT) depended on energy to remain oxidized and correctly folded, they apparently did not reach a stable minimum-energy-state. Such a state is probably attained only when the HA0 trimerizes and reaches the Golgi (U. Tatu, I.B., J.H. and A.H., unpublished result). We conclude that the ER lumen in the energized cell is a highly dynamic folding environment.

Protein folding in mitochondria and in the cytosol of bacteria is energy-dependent[14–17]. ATP is hydrolysed by chaperones that associate with the folding polypeptides and prevent nonproductive intra- and intermolecular interactions. For the ER, the situation has been less clear. A misfolded, temperature-reversible folding mutant, the ts045 G protein of vesicular stomatis virus, required ATP for its refolding after a shift from nonpermissive to permissive temperature[18], but we could not demonstrate a similar requirement for the wild-type protein. MHC class I antigen assembly *in vitro* is energy-dependent[19]. Evidence that ATP is present in the ER lumen came with the discovery of an ATP translocator in the ER membrane[20].

The most likely molecular explanation for the energy requirement is the involvement of BiP[9,21] in the post-translational folding process. BiP is an hsp70 analogue[22] generally assumed to be a folding factor in the ER[23–25]. It binds and hydrolyses ATP as it associates and dissociates from peptides and proteins[22,26]. The involvement of BiP is consistent with the observation that CCCP (carbonyl cyanide *m*-chlorophenyl hydrazone) prevents secretion of factor VIII and induces its accumulation in a complex with BiP[27]. We have been unable to detect BiP binding to normal folding intermediates of HA0 (Table 1), but this could simply mean that the associations are rapidly reversed or too ATP-sensitive to withstand the immunoprecipitation conditions. Another explanation for the observed energy-dependence of folding could be that energy is required to maintain oxidizing conditions inside the ER. We believe this not to be the case because ATP depletion did not lead to inhibition of disulphide bond formation, but rather to the formation of incorrect disulphide bonds. Apparently the oxidizing conditions in the ER were maintained (and re-established after DTT wash-out) in energy-depleted cells. We also observed HA0 misfolding during ATP-depletion even in the presence of DTT, that is, independent of disulphide bonds.

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