A novel system for continuous protein refolding and on-line capture by expanded bed adsorption

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Abstract

A novel two-step protein refolding strategy has been developed, where continuous renaturation-by-dilution is followed by direct capture on an expanded bed adsorption (EBA) column. The performance of the overall process was tested on a N-terminally tagged version of human β_2 -microglobulin (HAT-h β_2 m) both at analytical, small, and preparative scale. In a single scalable operation, extracted and denatured inclusion body proteins from *Escherichia coli* were continuously diluted into refolding buffer, using a short pipe reactor, allowing for a defined retention and refolding time, and then fed directly to an EBA column, where the protein was captured, washed, and finally eluted as soluble folded protein. Not only was the eluted protein in a correctly folded state, the purity of the HAT-h β_2 m was increased from 34% to 94%, and the product was concentrated sevenfold. The yield of the overall process was 45%, and the product loss was primarily a consequence of the refolding reaction rather than the EBA step. Full biological activity of HAT-h β_2 m was demonstrated after removal of the HAT-tag. In contrast to batch refolding, a continuous refolding strategy allows the conditions to be controlled and maintained throughout the process, irrespective of the batch size; i.e., it is readily scalable. Furthermore, the procedure is fast and tolerant toward aggregate formation, a common complication of in vitro protein refolding. In conclusion, this system represents a novel approach to small and preparative scale protein refolding, which should be applicable to many other proteins.

Keywords: protein refolding; expanded bed absorption (EBA); recombinant proteins; inclusion bodies

Heterologous protein production in bacteria has the potential to supply virtually unlimited amounts of high-value products. Unfortunately, the outcome is frequently unsatisfactory due to the deposition of recombinant

protein as inactive insoluble inclusion bodies, particularly when expression levels are high (Marston 1986). To recover the biological activity of the protein, extraction into denaturing buffer followed by in vitro refolding is necessary. Methods available for in vitro protein refolding are usually of low efficiency, and therefore, the high levels of bacterial expression do not readily translate into high yields of functional product.

During refolding, soluble and insoluble byproducts form as a result of inappropriate rearrangements within the protein itself and/or through unfavorable interactions with neighboring proteins. These interactions are typically mediated through exposed hydrophobic surfaces and/or interdisulfide cross-linking (Speed et al. 1996). Such inter-

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molecular aggregation processes are second or higher order reactions (Kiefhaber et al. 1991; Middelberg 2002). At high-protein concentrations, they dominate over productive folding, leading to decreased yields (Rudolph and Lilie 1996). To minimize adverse intermolecular interactions, the protein concentration should be kept low throughout the folding reaction, i.e., one is required to work with large reaction volumes. Even so, the formation of aggregates is difficult to avoid completely and the net result of an *in vitro* folding process is therefore likely to be a large reaction volume containing the diluted protein in a turbid suspension, a situation that severely hampers downstream processing, as several clarification steps are needed prior to fractionation by packed bed chromatography. Furthermore, the handling of large volumes increases the cost of industrial processes significantly.

Recent developments have therefore been aimed at establishing processes where refolding can be performed at high-protein concentrations (Zardeneta and Horowitz 1994a; Clark 1998). This includes batch and/or on-column refolding in the presence of enzymatic folding catalysts immobilized onto chromatographic supports (Stempfer et al. 1996; Altamirano et al. 1997; Dong et al. 2000). Possible folding catalysts include chaperonins, which bind to intermediate protein structures, thereby preventing aggregation; protein disulfide isomerases; and peptidyl-prolyl *cis-trans* isomerases, which promote disulfide bond formation and *cis-trans* isomerization, respectively, events that have often been identified as rate-limiting steps of the folding pathway (Jager and Pluckthun 1997; Rothwarf et al. 1998). Although these processes have facilitated the refolding of difficult-to-fold proteins (Altamirano et al. 1999, 2001), a number of problems persist that have prevented exploitation at large scale. These problems include (1) availability and cost of catalytic enzymes, (2) difficulty in regenerating the chromatographic support, as harsh conditions can lead to inactivation of the immobilized enzymes, and (3) scalability of the process. Furthermore, such assisted refolding might be limited to a narrow set of substrates, and conditions for optimal *in vitro* refolding might not be compatible with the activity of the catalytic enzymes.

On-column renaturation of protein is generally difficult to handle, as most *in vitro* refolding reactions are highly inefficient, resulting in both soluble and insoluble misfolded species, which could potentially foul the chromatographic adsorbents. Batch operations are not ideal either, as the conditions for folding-by-dilution are difficult to control at larger scales due to the problem of rapidly mixing large suspension volumes. The latter is particularly evident for folding reactions that proceed at rates in which the majority of the events (i.e., productive folding or aggregation) are completed within milli-

seconds to seconds (Goldberg et al. 1991; Dobson and Karplus 1999) i.e., faster than effective mixing can be achieved. Thus, there is a need to develop systems for protein folding, in which the conditions can be controlled and maintained throughout the reaction at both small and large scales of operation. In particular, the refolding reaction should be defined with respect to time in order to achieve high reproducibility and predictability of the protein yield from small- to large-scale processing. Moreover, the system should be robust toward insoluble protein aggregates, which might form during the course of the folding reaction.

Here, a novel process is presented, in which refolding can be carried out in continuous mode under controlled and specified conditions. This is obtained by diluting the denatured protein within a small flowthrough mixing chamber, in which the folding conditions can be carefully controlled and maintained throughout the entire process. From this mixing chamber, the proteins pass through a folding pipe reactor with sufficient retention time to allow folding. Finally, the nascently folded protein is directly captured by expanded bed adsorption (EBA)—a special type of fluidized bed chromatography that can handle crude suspensions and large reaction volumes (Draeger and Chase 1990, 1991; Thömmes 1997). Our approach uncouples the events of protein refolding and capture, thereby allowing each event to be optimized individually. The performance of the present process was tested with a crude inclusion body extract of N-terminally tagged human β_2 -microglobulin (HAT-h β_2 m), the light chain of the major histocompatibility complex class I (MHC-I) molecule.

Results

Production of denatured and correctly oxidized HAT-h β_2 m

HAT-h β_2 m was produced as insoluble inclusion bodies by *Escherichia coli* fermentations, and Figure 1A shows an SDS-PAGE analysis of samples collected before and after induction with IPTG. Expression of HAT-h β_2 m was detected 1 h after induction, and the level continued to increase until a maximum plateau was reached after ~2 h (Fig. 1A). Three hours after induction, the inclusion bodies were released by either enzymatic or mechanical disruption of the cells. The released inclusion bodies were washed and solubilized in 8 M urea under nonreducing conditions, yielding denatured and oxidized HAT-h β_2 m (Fig. 1B,C).

Correctly oxidized monomeric h β_2 m molecules contain two cysteine residues, forming a single disulphide bond, which can be demonstrated by comparing the mobility of the h β_2 m protein band under reducing and nonreducing

Figure 1. SDS-PAGE analysis of expression levels in *E. coli* and solubilized inclusion body preparations of HAT-h β_2 m. (A) Expression level of HAT-h β_2 m before and after induction. (Lane 1) Protein marker; (lane 2) before induction; (lanes 3–5) expression levels after 1, 2, and 3 h of induction, respectively. (B,C) Reducing and nonreducing SDS-PAGE analysis of feedstock A. (Lane M) Protein marker. DTT, dithiothreitol; 2-ME, 2-mercaptoethanol. Molecular weights of standard protein are indicated, and the positions of reduced (0) and oxidized (1) HAT-h β_2 m monomers are shown with arrows.

conditions in SDS-PAGE gels (Fig. 1B,C). The mobility shift of the reduced (Fig. 1B,C, band 0) and oxidized (Fig. 1B,C, band 1) HAT-h β_2 m bands is most clearly seen with DTT as the reductant (Fig. 1B). The kink in the HAT-h β_2 m protein band seen in Figure 1C is the result of diffusion of 2-ME from the reducing lane into the bordering nonreducing lane. This partial mobility shift of the HAT-h β_2 m band in the nonreducing lane confirms that the oxidized protein band (1) shifts into the reduced band (0) upon reduction. Monomeric HAT-h β_2 m was found to be fully oxidized in the feedstock preparations used in the following refolding experiments. The advantage of refolding correctly oxidized species is that the soluble target molecule can be generated in a simple and fast folding-by-dilution reaction without adding expensive redox pairs such as glutathione and oxidized glutathione to promote disulphide bond formation.

The purity of feedstocks A (enzymatic cell disruption) and B (mechanical cell disruption) with respect to oxidized HAT-h β_2 m monomers was 34.0% ($\pm 2.6\%$) and 56.0% ($\pm 1.3\%$), respectively, as determined by SDS-PAGE and densitometry. The total inclusion body protein recovery was 0.7 g/L and 3.0 g/L bacterial culture for feedstock preparations A and B, respectively.

Batch refolding studies

The impact of the total protein and urea concentration on the refolding yields of HAT-h β_2 m was investigated in analytical scale batch reactions. The formation of insoluble aggregates of HAT-h β_2 m in the protein concentration range of 10–1000 μ g total protein/mL was measured by

spectrophotometry at three different urea concentrations (Fig. 2). Aggregation could not be detected at OD_{450nm} when refolding was conducted in the protein concentration range of 10–100 μ g/mL, irrespective of the urea concentrations. However, the P_{sol}/P_{tot} ratio in this particular region decreased slightly (2%–3%) (Fig. 2), indicating that microaggregates had formed, and that these could be removed by centrifugation at 20,000g. Visible particulate aggregates started to appear when the protein concentration was raised to 200 μ g/mL during refolding at 100 mM urea. However, the refolding reaction could be completed without further aggregation at two- to three-fold higher protein concentrations when the level of urea was increased to 224 mM and 630 mM, respectively. Thus, aggregation of HAT-h β_2 m is highly dependent on the level of urea after folding by dilution. In all cases, the highest level of recovered soluble HAT-h β_2 m was observed at the lowest protein concentration, i.e., 10 μ g/mL (Fig. 2).

EBA-based system for continuous protein refolding and purification

A diagram of the system used for continuous protein refolding and on-line EBA capture is depicted in Figure 3. Protein refolding is initiated by dilution of the denatured protein suspension in a flowthrough mixing chamber. Refolding conditions (i.e., total protein concentration, urea concentration, and refolding time) can be set and maintained throughout the process by adjusting the flow rates of buffer and denatured protein suspension into the

Figure 2. Aggregation of HAT-h β_2 m as a function of the protein and urea concentration. Refolding was initiated by batch dilution in 20 mM Tris-HCl (pH 8.0), and the formation of particulate aggregates was measured by spectrophotometry at 450 nm after 30 min incubation at RT. The protein content of the samples was determined after centrifugation and the ratio of soluble (P_{sol}) and total protein (P_{tot}) was calculated. Filled symbols indicate OD_{450nm} measurements, and open symbols indicate P_{sol}/P_{tot} values at urea concentrations of 100 mM (squares), 224 mM (circles), and 630 mM (triangles), respectively. The data shown are the average of three independent folding reactions, and standard deviations are included at each point.

Figure 3. Schematic representation of the system for continuous protein refolding and on-line EBA capture. All EBA operations—i.e., equilibration, loading, washing, elution, and cleaning—were performed in expanded mode in Fastline10 and 50 columns. The current system allows the refolding buffer to be recycled through the system. Closed arrows indicate the direction of liquid flow during the folding/capture step, and open arrows indicate manual valves.

flowthrough mixing chamber and by changing the length of the folding-pipe reactor. Initial experiments were performed with feedstock A at 10 $\mu\text{g/mL}$ and 224 mM urea to achieve the highest possible recovery of HAT-h $\beta_2\text{m}$ and the least amount of insoluble aggregates (Fig. 2). The length of the folding-pipe reactor was adjusted to allow the protein 14 sec to fold. The intermediate urea concentration was selected to minimize the viscosity of the feed stream entering the EBA column.

The elution profile from the EBA column in expanded mode using a linear 0–1 M NaCl gradient together with a SDS-PAGE analysis of selected fractions are shown in Figure 4, A and B. It can be seen that soluble monomeric HAT-h $\beta_2\text{m}$ was eluted in a broad peak with an extensive tail (0.1–0.6 M NaCl). The majority of the soluble contaminants were eluted between 0.2 M and 0.4 M NaCl. The tailing of the HAT-h $\beta_2\text{m}$ peak is most likely the result of elution being performed in expanded mode. A second and sharper peak was detected later in the gradient, which could not be explained by the protein determination data or the SDS-PAGE analysis (Fig. 4, cf. A and B). However, fractions collected in this region also gave rise to an absorbance at 254 nm, indicating the presence of DNA and/or RNA in the samples. Agarose gel electrophoresis analysis followed by CYBR Gold staining confirmed that fractions 30 through 36 contained trace amounts of contaminating DNA and/or RNA (data not shown).

HAT-h $\beta_2\text{m}$ was not detected by SDS-PAGE in the flowthrough fractions (Fig. 4B, lane 4), demonstrating that all of the folded HAT-h $\beta_2\text{m}$ had adsorbed to the STREAMLINE DEAE medium. Purities of fractions enriched in monomeric HAT-h $\beta_2\text{m}$ were estimated by densitometric analysis and ranged from > 95% for the first fraction (Fig. 4B, lane 6) down to ~60% for fractions 27–30 (Fig. 4B, lanes 8–10). Although no visible aggregation (i.e., $\text{OD}_{450\text{nm}} = 0$) could be detected under the refolding conditions of this experiment (Fig. 2), > 50% of the total amount of monomeric HAT-h $\beta_2\text{m}$ applied to the system could not be accounted for in the linear elution with NaCl.

In order to increase the purity of the product peak, and to improve the control of the process at larger scales, the gradient profile was changed from linear to stepwise elution using a concentration of 0.15 M NaCl in the first step, followed by a second step with 1 M NaCl. To account for the protein that could not be desorbed from the EBA medium using 1 M NaCl, two additional elution steps under denaturing conditions (i.e., one without reducing agent, followed by one with reducing agent) were included.

Figure 4. Small-scale continuous refolding and EBA capture of HAT-h $\beta_2\text{m}$ using a 1-cm diameter EBA column with STREAMLINE DEAE operated in expanded mode. (A) Elution profile using a linear gradient of 0–1 M NaCl. Also shown is the protein concentration in the eluted fractions (■). (B) SDS-PAGE analysis of selected fractions from A. (Lane 1) Protein marker; (lane 2) reduced feedstock A; (lane 3) non-reduced feedstock A; (lane 4) flowthrough; (lanes 5–13) fractions 4, 7, 10, 13, 16, 19, 22, 24, and 26, respectively. (C) Elution profile using a stepwise gradient consisting of the following steps: (1) 0.15 M NaCl, (2) 1 M NaCl, (3) 1 M NaCl in 8 M Urea, and finally, (4) 1 M NaCl in 8 M Urea and 5 mM 2-ME as indicated above the chromatogram. (D) SDS-PAGE analysis of selected fractions from C. (Lane 1) Protein marker; (lane 2) reduced feedstock A; (lane 3) nonreduced feedstock A; (lane 4) flowthrough; (lanes 5–8) peaks 1, 2, 3, and 4 from C, respectively. Molecular weights of standard proteins are shown and the position of monomeric HAT-h $\beta_2\text{m}$ is indicated with arrows.

Table 1. Comparison of small and large scale processing

Scales of operation/Samples	Volume (mL)	Total protein (mg)	Total HAT-h β ₂ m (mg)	Concentration factor (fold)	Total recovery (%)	Purity (%)
Small scale						
Folded suspension	735 ^a	10.5	3.60	1.0	100.0 ^b	34.0 ($\pm$ 2.6) ^c
Peak 1	45	1.6	1.50	7.0	43.0	94.0
Peak 2	15	0.5	0.07	0.9	2.0	14.0
Large scale						
Folded suspension	18700 ^a	262.0	89.00	1.0	100.0 ^b	34.0 ($\pm$ 2.6) ^c
Peak 1	1530	44.0	37.00	5.0	41.0	83.0
Peak 2	925	33.0	1.30	0.4	1.5	4.0

No monomeric HAT-h β ₂m was detected in the unbound fraction (see Figs. 4B, 6B).

^a Calculated total volume after refolding at ~10 μ g total protein/mL.

^b Assuming a 100% refolding efficiency.

^c Purities of folded feedstock A represent an average of four independent measurements.

The elution profile using the stepwise gradient and the corresponding SDS-PAGE analysis of the peaks collected are shown in Figure 4, C and D. Highly purified soluble HAT-h β ₂m monomer (~94% purity) eluted in the first peak (Fig. 4C, step 1), whereas soluble contaminants together with minor amounts of HAT-h β ₂m (~2% of the total denatured amount applied to the folding reaction) were eluted in the second peak (Fig. 4C, step 2). A summary of the recovery data is presented in Table 1. Approximately 43% of the total amount of denatured monomeric HAT-h β ₂m offered to the refolding reaction could be recovered as monomeric HAT-h β ₂m under native elution conditions (Table 1; Fig. 4C, peaks 1,2), and the target protein was concentrated sevenfold in the first peak. The remaining HAT-h β ₂m protein could only be eluted under denaturing and reducing conditions (Fig. 4C,D, peaks 3,4). Furthermore, the SDS-PAGE analysis of peaks 3 and 4 demonstrate that a large amount of contaminating proteins, which apparently remained soluble during the refolding reaction, had adsorbed to the medium in a state that precluded their elution under native conditions (Fig. 4D, lanes 7,8). Note that the high degree of aggregation of the target molecule and other proteins did not, at any stage, affect the performance and stability of the expanded bed, i.e., no channels or stagnant zones were observed, and the bed height remained constant throughout the loading/refolding step. After elution with urea and re-equilibration with binding buffer (i.e., 20 mM Tris-HCl [pH 8.0]), the bed expanded to the initial height, indicating that the medium could be regenerated.

Investigation of refolding time, efficiency, and system robustness

Although the refolding reaction in the previous experiments was conducted under conditions that did not lead to aggregation as measured by OD_{450nm} (Fig. 2), a significant

amount of the presumably folded HAT-h β ₂m was adsorbed to the EBA medium in a form that could only be released by renewed denaturation (Fig. 4; Table 1). This loss might be the result of insufficient refolding time (i.e., 14 sec) in the pipe reactor, which produced an immature protein that was prone to aggregation or unfolding on the chromatography medium. To further investigate the importance of the refolding time on the total recovery of soluble monomeric HAT-h β ₂m, a pipe reactor was inserted between the flowthrough mixing chamber and the EBA column, allowing the protein to fold for 10 min before EBA capture. Moreover, in a batch-refolding experiment conducted under the same conditions, the protein was allowed 30 min to fold before starting to load the suspension onto the EBA column. Due to limited availability of feedstock A, these experiments were conducted with feedstock B, and in all experiments, the EBA column was washed and eluted with a step gradient of 1 M NaCl in a similar way to that shown in Figure 4C.

Despite the increase in refolding time, the resulting yield of soluble monomeric HAT-h β ₂m was only marginally improved (53% using 14-sec refolding time vs. 57.6% and 56.7%, using 10- and 30-min refolding times, respectively). This indicates that the refolding reaction for HAT-h β ₂m had essentially run to completion within 14 sec and had left a significant fraction of soluble monomer forms that could not be recovered under native elution conditions in the subsequent EBA chromatography.

In light of the above results, the possibility that some folded and mature HAT-h β ₂m became irreversibly bound to the EBA support due to, for example, unfolding or aggregation, was examined. Refolded monomeric HAT-h β ₂m, which had already gone through one complete refolding-EBA purification cycle as shown in Figure 3, was desalted by Sephadex G-25 chromatography to remove the NaCl used during elution and then reapplied to the EBA column. To mimic the refolding conditions,

the desalted HAT-h β_2 m pool was diluted in binding buffer (20 mM Tris-HCl [pH 8.0] without urea) to a final protein concentration of 10 μ g/mL prior to the EBA-loading step. In this case, > 99% of the applied HAT-h β_2 m was recovered in the eluted fraction using 1 M NaCl, and only ~0.1% appeared in the 8-M urea cleaning steps (Fig. 5). Thus, the observed loss during continuous refolding and direct EBA capture can be attributed to the refolding reaction and not the chromatographic operation itself. It could be reasoned that the refolding reaction produces at least two soluble monomeric HAT-h β_2 m populations. One population is correctly refolded and fully recoverable; the other population is misfolded and interacts with the STREAMLINE DEAE medium in a way that leads to aggregation. Alternatively, a large amount of microaggregates are generated during the refolding reaction, which adsorbs strongly to the chromatographic medium.

Finally, the performance of the system for continuous refolding and direct EBA capture was tested at higher protein concentrations during refolding. Continuous refolding was conducted at 200 and 600 μ g total protein/mL at a urea concentration of 224 mM urea. In both cases, the same amount of total protein was applied to the EBA column as for the 10 μ g/mL experiment. Importantly, refolding at 600 μ g/mL lead to the formation of insoluble aggregates (see also Fig. 2). Adsorbed proteins were eluted with 1 M NaCl in binding buffer, and the aggregated

proteins were recovered using 8 M urea in binding buffer as described above. The recovery data are compared in Figure 5. In all cases, soluble monomeric HAT-h β_2 m could be eluted from the EBA column. Not surprisingly, the total recovery of soluble monomeric HAT-h β_2 m decreased from 53% to 16% when the protein concentration during refolding was raised from 10 to 600 μ g/mL as a result of the decreased refolding efficiency and formation of insoluble aggregates (Fig. 5). Compared with the initial continuous refolding experiments conducted with feedstock A at 10 μ g/mL, the total recovery of monomeric HAT-h β_2 m was increased by ~10% with feedstock B (cf. Fig. 5 and Table 1). Feedstock B had a higher purity than feedstock A, raising the possibility that the presence of contaminating proteins affects the refolding efficiency of HAT-h β_2 m negatively.

No bed stability problems were observed visually during loading of the EBA column when the continuous protein refolding reaction was performed at 10 and 200 μ g protein/mL, even though > 50% of the monomeric HAT-h β_2 m was adsorbed to the chromatographic medium in an insoluble state (Figs. 4, 5). Although insoluble aggregates were seen breaking through the column when refolding was conducted at 600 μ g protein/mL, some channeling and stagnant zones were observed at the bottom part of the bed (i.e., between 1 and 5 cm). The channeling effects were not very severe and did not lead to breakthrough of soluble HAT-h β_2 m as judged by SDS-PAGE analysis of the flowthrough fraction. Since the instabilities in the bed disappeared during the elution phase with NaCl, they can most likely be attributed to electrostatic interactions between the soluble and insoluble protein aggregates formed at 600 μ g/mL and the DEAE ligands on the support surface causing cross-linking of neighboring beads. The bed stability problems observed at 600 μ g/mL might be partly resolved by increasing the urea concentration during refolding from 224 to 630 mM, as the degree of aggregate formation was decreased by approximately sevenfold at the latter condition (Fig. 2).

Figure 5. Investigation of HAT-h β_2 m monomer recovery after continuous refolding at different protein concentrations and EBA purification. The total recovery following elution of previously refolded and purified HAT-h β_2 m, which was reloaded onto the EBA column, is shown in the first column. No soluble monomeric HAT-h β_2 m was detected in the flowthrough fraction in any of the four experiments, and equal amounts of total protein (~10 mg) were applied to the column. (Filled bars) Soluble monomeric HAT-h β_2 m eluted with 1 M NaCl in 20 mM Tris-HCl (pH 8.0); (open bars) insoluble monomeric HAT-h β_2 m eluted under denaturing conditions (8 M urea, 1 M NaCl in 20 mM Tris-HCl [pH 8.0]).

Scalability of the system

The scalability of the present process was investigated by increasing the amount of feedstock processed 25-fold through the use of a 5-cm diameter EBA column. Conditions applied for the scaled-up process during refolding (i.e., total protein concentration, urea concentration, and retention time in the pipe reactor) and EBA operation (i.e., linear fluid velocity, elution conditions, and total load of protein per volume of adsorbent) were kept constant. Refolding conditions were 10 μ g/mL, 224 mM urea using a folding time of 14 sec in the pipe reactor. Although, as discussed above, slightly higher recoveries

Figure 6. Preparative scale continuous refolding and direct EBA capture of HAT-h β_2m . (A) Elution profile in expanded mode from a 5-cm diameter EBA column using a step gradient of 0.15 M NaCl, followed by 1 M NaCl. (B) SDS-PAGE analysis of peaks collected. (Lane 1) Protein marker; (lane 2) reduced feedstock A; (lane 3) nonreduced feedstock A; (lanes 4,5) peaks 1 and 2, respectively; (lane 6) supernatant from support sample incubated with 8 M urea, 1 M NaCl, 10 mM 2-ME in 20 mM Tris-HCl (pH 8.0) overnight. Molecular weights of standard proteins, and the position of monomeric HAT-h β_2m is shown with an arrow.

could be obtained with a longer retention time in the pipe reactor, 14 sec was selected to keep the system as simple as possible at larger scale. The elution profile obtained at preparative scale (Fig. 6A) was similar to that observed for the small scale process (Fig. 4C), and SDS-PAGE analysis of collected fractions showed that highly purified soluble monomeric HAT-h β_2m was eluted in the first peak (Fig. 6B). At preparative scale, the 8-M urea elution steps were omitted, and instead, the medium was cleaned by pumping 1–2 CVs of 1 M NaOH through the system, followed by recycling of the cleaning agent overnight. Prior to cleaning, a sample of support was removed from the column and treated with 8 M urea, 1 M NaCl, supplemented with 10 mM 2-ME overnight at 4°C. SDS-PAGE analysis of the supernatant showed that a large amount of HAT-h β_2m and contaminating proteins had adsorbed to the medium in an insoluble state (Fig. 6B), similarly to the scenario observed at small scale processing (Fig. 4D).

The recoveries for small and preparative scale operation are summarized and compared in Table 1 for feedstock A. Very similar total recoveries of soluble monomeric HAT-h β_2m were obtained, but the purity and concentration of the target protein was slightly lower at preparative scale. The latter is the result of instability in the expanded bed caused mainly by the rotating distributor system and the presence of coalesced bubbles arising from dissolved air in the buffers, which necessitated positioning the top adaptor ~10 cm above the expanded bed to avoid chromatographic beads being flushed from the column. By comparison, the top adaptor was placed ~4 cm above the expanded bed during the elution procedure at small scale. Hence, the lower concentration factor at preparative scale in this particular case is a direct result of the increased liquid headspace above the expanded bed, leading to dilution of the

product peak. In a similar refolding and capture experiment using a STREAMLINE 50 EBA column, these problems were not observed (data not shown), and the decrease in concentration factor shown in Table 1 therefore does not affect the general conclusion that the process is scalable.

Determination of the biological activity of HAT-h β_2m and h β_2m

For the small-scale preparation, the recombinant h β_2m was released from the refolded and purified HAT-h β_2m molecule by FXa cleavage, and the resulting peptide tag and undigested product were removed by Ni-NTA chromatography as described in Materials and Methods. h β_2m was collected in the flowthrough fraction, concentrated, and further purified by size-exclusion chromatography on Sephadex G-50 to remove FXa and peptides.

The ability of the MHC-I heavy chain to bind peptide is completely dependent on the presence of correctly folded h β_2m (Garboczi et al. 1992). The functionality of the recombinant h β_2m was therefore tested in a peptide-MHC-I binding assay. Figure 7 compares the h β_2m dose-response data of three different versions of h β_2m : (1) h β_2m purified from natural sources; (2) folded, released, and purified recombinant h β_2m ; and (3) folded HAT-h β_2m . Peptide binding was detectable at h β_2m concentrations < 10 nM, which is comparable to what has previously been reported for recombinantly produced h β_2m (Pedersen et al. 1995, 2001). The biological activity of the natural h β_2m and the recombinant h β_2m were essentially identical, demonstrating that correctly folded h β_2m can be generated using the EBA-based system for continuous refolding presented here.

In contrast, the HAT-h β_2m was less potent. Peptide binding became detectable around 50 nM HAT-h β_2m ,

Figure 7. Determination of the biological activity of refolded and purified hβ₂m (filled symbols) and HAT-hβ₂m (open symbols) using a biochemical peptide-MHC-I binding assay. Denatured truncated heavy chain HLA-A*1101 (3 nM) was folded by dilution in a buffer containing different concentrations of hβ₂m and HAT-hβ₂m, respectively, and a fixed amount of a specific radiolabeled peptide (~1–3 nM). (■) Native hβ₂m, standard; (●) recombinant hβ₂m, small scale; (○) HAT-hβ₂m, small scale; (△) HAT-hβ₂m, preparative scale. The difference between duplicate peptide-binding measurements were within 5%, and the solid line through the data represents a nonlinear regression analysis using the four-parameter logistic function of the SigmaPlot 8.0 program (*R*² values were within 0.994–0.999). The inset shows a nonreducing SDS-PAGE analysis of hβ₂m before and after FXa cleavage. (Lane 1) Protein standards; (lane 2) HAT-hβ₂m sample; (lane 3) hβ₂m sample.

and ~10-fold higher amounts of HAT-hβ₂m were needed to reach the same degree of binding as with hβ₂m. Moreover, the level of maximum binding is not the same for hβ₂m and HAT-hβ₂m, indicating that association of HAT-hβ₂m with the heavy chain changes the overall affinity of the receptor for peptide binding. A changed affinity could reflect a distortion of the MHC-I structure due to the steric hindrance imposed by the long N-terminal HAT-tag.

Finally, we tested the activity of HAT-hβ₂m produced at small and preparative scale and found no difference, indicating that the product obtained was qualitatively the same at both scales of operation (Fig. 7).

Discussion

In vitro protein refolding reactions are generally of very low efficiency, resulting in the formation of both soluble and insoluble misfolded forms of the target molecule, which in the subsequent downstream processing steps must be separated from the native protein using a range of technologies including clarification steps and chromatography. It is well known that the reaction can be

skewed toward productive refolding by keeping the protein concentration low (i.e., 10–50 μg/mL) throughout the course of the reaction (Rudolph and Lilie 1996; Clark 2001). However, the latter produces a situation where excessive reaction volumes (e.g., 5000–200,000 liters, depending on the target protein) are needed to produce high product recoveries, and such processes are difficult to handle at larger scales, requiring special holding tanks and large mixing reactors (Gallagher 1991; Wheelwright 1991). The yield of the folding reaction is dependent not only on the protein concentration, but also on the chemical and physical composition of the environment and the time that the target molecule is given to reach its native conformation. Thus, to predict the outcome of a given refolding reaction, defined and controlled conditions are required. The latter is very difficult, if not impossible, to achieve with conventional batch folding-by-dilution reactions, as homogenous mixing conditions cannot be obtained rapidly enough for protein-refolding reactions (Lee et al. 2002). Thus, gradients of protein and denaturant concentrations, pH, and ionic strength are produced during the initial stages of the refolding reaction. Moreover, the refolding time cannot be defined, as it takes time to transfer the suspension from the mixing reactor to the next step, e.g., column chromatography, leading to a situation in which different protein molecules experience varying folding times and environments, which will affect the overall yield of the reaction.

Others have attempted to resolve these issues by exploiting continuous stirred reactors (Gallagher 1991), where the refolding is performed in a smaller reaction volume than under batch operations. Although the mixing conditions might be considerably improved in such a process, the time for refolding can still not be precisely specified and controlled, as the first denatured molecule that enters the reactor could theoretically also be the last to leave. Thus, only average refolding times can be given. In another study, oscillatory flow reactors were used to produce “ideal” mixing conditions for in vitro protein refolding (Lee et al. 2002). However, when compared with conventional stirred-tank reactors, the authors were unable to demonstrate higher recoveries using the oscillatory flow reactor. Furthermore, the use of such a mixing design cannot solve the critical problem of defining and controlling the refolding time, as time is required to move the suspension from the reactor.

In two completely different studies, the EBA technology was used to develop processes for matrix-assisted refolding of human growth hormone (Cho et al. 2002) and green fluorescent protein (Cabanne et al. 2005). In these studies, the target protein was bound to the adsorbent through a tag-sequence and under denaturing conditions. Subsequently, the folding reaction was initiated by

pumping aqueous buffer into the column. In the former study, the folding reaction was initiated in expanded-bed mode, whereas in the latter study, the folding reaction was initiated in settled-bed mode. In any event, these approaches fail to define and control the folding conditions, as a gradient of protein concentrations will exist throughout the length of the column, the highest concentration being found at the inlet, where the EBA support will become saturated with protein. Furthermore, misfolded and aggregated species formed during the reaction will most likely bind to the adsorbent as the refolding is initiated with the protein immobilized on the support. None of the EBA-based studies for matrix-assisted folding have addressed the issues of cleaning-in-place.

An inherent weakness of the fused-process approach of matrix-assisted refolding has to include the need for low-fluid velocities to avoid excessive bed expansion in the EBA column and density-induced mixing issues. Both of these are direct consequences of using a chaotrope containing feedstock and their impact on processing speed and scalability, which cannot be underestimated. A third and equally important consequence of using a chaotropic feedstock is that the molecular diameter of the denatured species is larger than its native counterpart. High levels of chaotropes add yet another degree of difficulty to purification of proteins by increasing the effective size of the molecule (Haynes and Norde 1994; Heebøll-Nielsen et al. 2003). This, in turn, leads to reduced access to the functionalized interior of the porous expanded-bed support, leading to slower binding kinetics and limited utilization of the available binding capacity of the support (Choe 2002; Heebøll-Nielsen et al. 2003). This reduction in available sorption performance (both kinetics and capacity), combined with the use of restricted fluid-flow rates, leads us to suggest that for a given system, the overall process productivity, i.e., the amount of purified folded product produced per unit time will be less for the coupled versus the uncoupled approach, as described herein.

Here, we describe an EBA-based process for continuous protein refolding, which can solve all of the previously mentioned problems. Continuous refolding-by-dilution is achieved by pumping the denatured protein suspension and the aqueous buffer through a very small flowthrough mixing chamber, allowing the folding reaction to be initiated instantaneously and reproducibly at both small and preparative scale operations without generating concentration gradients that affect the yield of the folding reaction (Fig. 3; Table 1). Moreover, the system ensures that all denatured protein molecules that enter the pipe reactor experience the same refolding environment and time before capture on the EBA support. For industrial-scale applications, the simple flowthrough mixing chamber and pipe reactor exploited in this study could be exchanged for commercial in-line

static mixers with completely defined flow profiles throughout the length of the pipe. In addition, the combination of continuous refolding and EBA capture allows the system to handle large volumes of crude suspensions at high flow rates with minimal hold-up time, as the nascently folded product is directly recovered on the EBA column after the refolding event. The latter reduces the risk of inadvertent modifications of the protein product, such as proteolytic degradation.

The performance of the system presented was tested on a tagged version of h β_2 m, i.e., the light chain of the MHC-I receptor, termed HAT-h β_2 m. The protein was produced as correctly oxidized denatured monomers (Fig. 1B,C) in line with previous work for generating the heavy-chain part of the MHC-I receptor (Ferré et al. 2003). In this study, a retention time in the folding-pipe reactor of 14 sec appeared to be sufficient to allow essentially complete refolding of the correctly oxidized monomeric HAT-h β_2 m molecules, since increasing the time to 10 and 30 min, respectively, only increased the recovery of HAT-h β_2 m by ~4%. The time required for folding of HAT-h β_2 m observed in this study is consistent with the results reported on refolding of oxidized h β_2 m by Chiti et al. (2001), which demonstrated that more than 90% of the structure forms within a time period of 5 sec as measured by stopped-flow intrinsic fluorescence.

Larger and more complicated proteins than HAT-h β_2 m might require longer refolding times and more complex buffer conditions to reach a stable and properly folded structure. In this case, the need to specify and control the conditions and time for refolding might be even more important than for the protein used in the present system. The *in vitro* refolding efficiency of more complex proteins is typically optimized and improved through the addition of additives such as detergents and redox pairs to minimize aggregation and promote disulphide bond formation, respectively (Zardeneta and Horowitz 1994a,b; Creighton et al. 1995). In the current system, such additives could be added directly to the refolding reaction through separate inlets in the flowthrough mixing chamber. In this context, it should be mentioned that the set-up (refer to Fig. 3) allows for reuse of the refolding buffer, thereby potentially salvaging expensive additives.

Interestingly, the purity of the denatured HAT-h β_2 m inclusion-body preparation appeared to effect the overall yield of the process, as increasing purity of the starting material from 34% (feedstock A) to 56% (feedstock B) increased the yield from ~45% to ~55%. Maachupalli-Reddy et al. (1997) recently found that contaminants in the inclusion-body preparation reduced refolding yields, whereas others have reported that contaminants do not affect refolding yields (Rudolph and Lilie 1996). These discrepancies may be linked to the particular refolding

protein molecule and the specific composition of the inclusion-body preparation. Interestingly, some of the work conducted on matrix-assisted folding using EBA by Cho et al. (2002) and Cabanne et al. (2005) has involved unclarified feedstocks. Although in this report we have used clarified feedstock preparations, we envision that it would also be possible to use unclarified feedstock. This would have the advantage of further reducing the total number of unit operations necessary to renature, capture, and purify HAT-h β_2 m with a concomitant reduction in production time and cost.

In conclusion, a scalable process for continuous protein refolding and direct EBA capture has been developed and successfully tested in this study using HAT-h β_2 m as a model. The system integrates in vitro refolding, capture, clarification, concentration, and initial purification into a single unit operation, and it is compatible with protein aggregate formation. The process is routinely used in our laboratories and has recently been applied to generate functional interleukin-2 and granulocyte colony stimulating factor, and we believe that it could be of benefit to the refolding of many other proteins.

Materials and methods

Cloning and expression of HAT-h β_2 m

The pT7H6-h β_2 m vector, which encodes a hexa-histidine tag (His₆-tag) followed by a FXa cleavage site (single letter amino acid code IEGR) and fused to the N terminus of the native h β_2 m, has been described previously (Pedersen et al. 1995). Here, the hexa-histidine tag has been replaced by a more soluble 19 amino acid histidine affinity tag (HAT) sequence (single letter amino acid code KDHLIHNHVKKEEHAHA HNK) (BD BioSciences). The fragment encoding the His₆-tag was excised using NdeI–BamHI restriction enzymes and replaced by a HAT-encoding duplex formed by the oligonucleotide primers: taccatgggcAAGGATCATCTgATCCACA ATGTCCACAAAGAGGAGCACGCTCATGCCCAAC AAGg and ggtaccgcTTCCTAGTAGAcTAGGTGTTACAGG TGTTTCTCCTCGTGCAGTACGGGTGTTGTTCCctag (the underlined cohesive ends are complementary to the NdeI and BamHI insertion site of the digested pT₇H₆-h β_2 m vector). The construct comprising MG-HAT-GS-FXa-h β_2 m was excised using NcoI and HindIII and inserted into the pET28a+ vector (Novagen) containing the kanamycin resistance gene, and subsequently transformed into *E. coli* strain BL21(DE3). Clones, which produced protein upon induction with IPTG, were identified and the inserted gene sequence was verified by DNA sequencing (ABI310, Perkin Elmer). Expression was done in a 2-L Labfors fermentor (Infors) by IPTG induction as previously described by Ferré et al. (2003).

Isolation and solubilization of inclusion bodies

Two batches of HAT-h β_2 m-derived inclusion body preparations were prepared and designated feedstock A and B. Isolation and solubilization of inclusion bodies leading to feedstock

A was performed as described by Ferré et al. (2003). Briefly, inclusion bodies were released with lysozyme and coreleased DNA and RNA were digested with DNase I and RNase A. Inclusion bodies were collected by centrifugation, washed, and finally solubilized in 8 M urea, 20 mM Tris-HCl (pH 8.0). For preparation of feedstock B, a 2-L fermentor culture was taken directly after a 3-h induction period and passed once through a cell disruptor at 2.5 kbar (model Basic Z plus, Constant Systems Ltd.). Inclusion bodies were collected by centrifugation at 17,000g for 10 min at 4°C, and subsequently washed according to the procedure described by Ferré et al. (2003). Washed inclusion bodies were solubilized in 8 M urea, 20 mM Tris-HCl (pH 8.0) by overnight incubation at 4°C. Insoluble material was removed by centrifugation at 17,000g for 10 min at 4°C, and the supernatant was filtered (0.45 μ m pore size). To minimize batch variability between refolding experiments, aliquots of filtered supernatants of 1 and 20 mL were stored at –20°C and used for the analytical, small, and preparative scale experiments, respectively.

Batch refolding

Analytical-scale batch refolding was performed in triplicate by dilution of denatured feedstocks with refolding buffer (20 mM Tris-HCl [pH 8.0]) in 2-mL Eppendorf tubes in a total reaction volume of 1 mL. Experiments at 10 μ g/mL were performed in a 2-mL reaction volume to provide enough protein for the subsequent analysis. Each reaction mixture was incubated for 0.5 h at room temperature (RT) on a vertical rotating mixer. Insoluble aggregates were removed by centrifugation at 15,000g for 10 min at 4°C, and the resulting supernatants were then analyzed for total soluble protein and purity using a BCA protein assay (see below) and SDS-PAGE, respectively.

Preparative batch refolding was initiated by adding 20 mL denatured feedstock into 700 mL binding buffer, resulting in a final urea concentration of 222 mM. The reaction was stirred using a magnetic rod (200 rpm) for 0.5 h at RT before the suspension was loaded onto the EBA column.

Small-scale continuous refolding and EBA capture

A 1.5-mL mixing chamber was made of a teflon housing and equipped with separate inlets for buffer and protein sample on opposite sides and one outlet at the top (MicroLab). To ensure instant mixing between the two liquid streams, the housing contained a magnetic rod, which was rotated at high speed, and the buffer inlet was angled in such a way that it supported the turning direction of the magnetic rod. Various lengths of silicone tubing could be placed between the outlet of the mixing chamber and the inlet of the EBA column. The outlet flow rate and the length of tubing determined the time between leaving the mixing chamber (i.e., initiating the folding step) and arriving at the EBA column (i.e., the capture step). For the small-scale experiments, the length of the pipe reactor was adjusted to allow the protein a refolding time of either 14 sec or 10 min prior to the capture step. The EBA column used was a 1-cm diameter Fastline10 (Upfront Chromatography). The distribution system of the Fastline columns is based on local magnetic stirring at the column inlet, and in contrast to other column designs, they do not have filter meshes at the inlet or at the top adaptor. EBA capture, elution, and cleaning was performed in expanded mode. To prevent the liquid from rising above the top adaptor in the EBA column due to pressure

drops in the tubing and in the UV flow cell, a glass tube was fused through the top of the outer glass column, allowing the air head space to be pressurized, using a syringe or a pump. The Fastline10 column was connected to a FPLC system (Amersham Biosciences), which controlled the flow of the refolding buffer and the collection of fractions. A peristaltic pump (model P1, Amersham Biosciences) was carefully calibrated (i.e., average flow rate based on three independent calibrations) and used to control the flow of denatured protein into the mixing chamber. The outlet of the EBA column was connected to an UV monitor (Uvicord S II, Amersham Biosciences), equipped with a 280-nm filter and an industrial flow cell. A REC 112 chart recorder (Amersham Biosciences) was used to log the UV signal. Buffers were filtered and degassed, and all experiments were performed at RT.

The Fastline 10 EBA column was filled with ~9 mL STREAMLINE DEAE medium (Amersham Biosciences) corresponding to a settled bed height of ~11 cm. Expansion/equilibration was done by pumping binding buffer (20 mM Tris-HCl [pH 8.0]) through the mixing chamber and onto the column at a flow rate of 2.5 mL/min (191 cm/h) until the bed height was constant and the bed appeared stable by visual inspection (approximately five expanded-bed CVs). When the bed was fluidized, the rotation of the magnetic rod at the column inlet was started at a speed of 400 rpm/min. The bed expanded to a height of 20 cm (corresponding to a twofold expansion) and the adaptor was positioned at 30 cm during the loading phase. During elution, the adaptor was lowered to ~4 cm above the expanded bed.

Refolding/loading was initiated by pumping crude solubilized inclusion-body derived HAT-h β 2m into the mixing chamber at a flow rate of 0.07 mL/min, while keeping the flow rate of the binding buffer constant at 2.5 mL/min. Unbound proteins and aggregates were removed by pumping binding buffer through the column until the A_{280nm} signal reached the baseline. Unbound proteins were collected in a single pool and adsorbed proteins were eluted with either a linear gradient of 0–1 M NaCl in binding buffer (developed over 10 expanded-bed column volumes) or a stepwise NaCl gradient: (1) 0.15 M NaCl in 20 mM Tris-HCl (pH 8.0) and (2) 1 M NaCl in 20 mM Tris-HCl (pH 8.0).

After the elution procedure, the flow rate was decreased to 1.5 mL/min (115 cm/h) and the medium was cleaned with 8 M urea, 1 M NaCl in 20 mM Tris-HCl (pH 8.0), followed by 8 M urea, 1 M NaCl, 5 mM 2-mercaptoethanol (2-ME) in 20 mM Tris-HCl (pH 8.0). The denaturing buffers used for cleaning were supplemented with 1 M NaCl to prevent rebinding of the HAT-h β 2m molecules upon solubilization in urea. Each elution/cleaning step was continued until the A_{280nm} signal reached the baseline. During the cleaning procedure, the bed height increased to ~25 cm due to the high viscosity of the buffer. Fractions of 5 mL were collected throughout all of the elution and cleaning steps except during the linear gradient elution, when fractions of 3 mL were collected. Selected fractions were assayed for protein content using the BCA assay and SDS-PAGE and the presence of copurified DNA/RNA was detected by A_{254nm} measurements.

Preparative scale refolding and EBA capture

For preparative refolding and EBA capture, the mixing chamber inlets were connected to a Masterflex L/R pump equipped with an Easy Load II pump head (Cole-Palmer) and a P1 peristaltic pump, respectively. A 5-cm diameter Fastline 50

column was filled with 230 mL STREAMLINE DEAE corresponding to a bed height of ~11.5 cm and expanded with refolding buffer at 60 mL/min (183 cm/h). The rotation rate of the magnetic rod at the column inlet was set at ~30 rpm. The bed expanded to 36 cm, and the top adaptor was set at 49 cm during loading. Refolding was initiated by pumping crude solubilized inclusion body-derived HAT-h β 2m into the mixing chamber at a rate of 1.7 mL/min while keeping the flow rate of the refolding buffer (i.e., 20 mM Tris-HCl [pH 8.0]) constant at 60 mL/min. After refolding, the column was washed with five expanded-bed CVs of refolding buffer. Elution was done in two steps: (1) 0.15 M NaCl in 20 mM Tris-HCl (pH 8.0) and (2) 1 M NaCl in 20 mM Tris-HCl (pH 8.0), and peaks were collected manually. Prior to cleaning, ~10 mL medium was removed from the bottom part of the column and a sample of 3 mL sedimented medium was treated overnight at 4°C with 17 mL of 8 M urea, 1 M NaCl, and 10 mM 2-ME in 20 mM Tris-HCl (pH 8.0). Cleaning of the EBA column was done by pumping 1–2 expanded CVs of 1 M NaOH through the bed at a flow rate of 50 mL/min (150 cm/h), followed by recycling of the remaining NaOH solution (8 L) overnight (~16 h). The column was then re-equilibrated with 20 mM Tris-HCl (pH 8.0).

Removal of HAT-tag and purification of h β 2m

Fractions from continuous refolding and EBA capture containing HAT-h β 2m were pooled and concentrated on a 3-kDa NMWL filter (Millipore) in a stirred nitrogen pressure cell (Amicon) to about 10 mL, and then adjusted to a final volume of 15 mL and final concentrations of 25 mM Tris-HCl (pH 8.0), 100 mM NaCl, 1 mM CaCl₂, and 0.1 mM NiSO₄. Factor Xa (Protein Engineering) was added to a final concentration of 1 μ g/mL, and the mixture was incubated at RT for at least 2 d. Progression of the cleavage reaction was monitored by SDS-PAGE. The digest was subsequently applied at 1 mL/min to a column (2.6 $\times$ 13 cm) containing Ni-NTA medium (Amersham Biosciences), during which undigested protein and the released tag peptide were removed. The flowthrough fraction containing refolded h β 2m was collected and concentrated to a final volume of 13 mL as described above. High molecular weight contaminants including multimerized h β 2m and FXa were removed by size-exclusion chromatography on Sephadex G-50 medium. The Sephadex column (2.6 $\times$ 100 cm) was equilibrated with 20 mM Tris-HCl (pH 8.0), and the fractionation was run at 0.8 mL/min. Highly purified monomeric h β 2m was collected in 10-ml fractions and pooled for further analysis.

Peptide-MHC-I binding assay

The function of β 2m is to support the ability of MHC-I heavy chain to bind antigenic peptides. The functionality of refolded and purified HAT-h β 2m, and of FXa released h β 2m was therefore determined in a biochemical peptide-MHC-I binding assay as described in Pedersen et al. (1995). Denatured MHC-I heavy chain (truncated HLA-A*1101, 3 nM;) (Ferré et al. 2003) was folded by dilution into buffers containing different concentrations (0–2000 nM) of HAT-h β 2m (or h β 2m) and a specific radiolabeled peptide (1–3 nM, single-letter amino acid code = KLFPPPLYLR). The refolding buffer was 100 mM Tris-maleate (pH 6.6) in PBS supplemented with 1 mg/mL pluronic copolymer Lutrol F-68 (BASF), and the reaction (100 μ L) was allowed to proceed for ~24 h at 18°C. Duplicate samples (15 μ L) of each folding reaction were sub-

sequently analyzed by Sephadex G-50 spun column chromatography as described by Buus et al. (1995). The radioactivity of the excluded "void" volume, containing formed MHC-I complexes, and that of the retained volume, containing unbound peptide, was measured by gamma spectrometry (Packard Instruments). Binding values were calculated by dividing excluded radioactivity with the total amount of radioactivity offered. The peptide-binding values were plotted as a function of the concentration of HAT-h β_2 m (or h β_2 m) in the folding reaction, and the data were fitted to a sigmoid dose-response curve using the four-parameter logistic function in the SigmaPlot 8.0 software package (SPSS Inc.).

Protein determination

The total amount of protein in feedstocks and collected fractions was determined using a BCA protein assay (Pierce) run on a COBAS MIRA spectrophotometer robot (Roche). Standard curves were constructed with BSA in the concentration range of 0–2 mg/mL in refolding buffer, i.e., 20 mM Tris-HCl (pH 8.0), and cleaning buffer, i.e., 8 M urea in binding buffer, to allow protein determination under both aqueous and denaturing conditions.

Electrophoresis

One-dimensional SDS-PAGE was performed with precast 4%–12% gradient NuPAGE gels (Laemmli 1970) using the 2-(N-morpholino) ethane sulfonic acid (MES) running buffer system according to the manufacturer's instructions (Invitrogen). Protein bands were stained with Coomassie Brilliant Blue. SDS-7 (Sigma) or Mark12 (Invitrogen) was used as protein standard on the SDS-polyacrylamide gels. If necessary, samples were concentrated in centrifugal filters equipped with 5-kDa cutoff membranes (Millipore) prior to SDS-PAGE analysis.

The presence of inclusion body-derived HAT-h β_2 m in collected fermenter samples was determined by SDS-PAGE analysis using the procedure described by Chen and Christen (1997).

Densitometry

Densitometric analysis was performed on nonreducing, Coomassie Brilliant Blue stained gels, using the Gel Doc 2000 documentation system (Bio-Rad) and the Quantity One software package (Bio-Rad). The purity of oxidized monomeric HAT-h β_2 m in the denatured feedstocks and collected fractions was calculated by relating the detected pixels in the monomer band to the accumulated amount of pixels found in the other detected bands or in the entire lane. The latter method was used to determine the purity in fractions, which upon SDS-PAGE analysis, exhibited band smears due to the presence of multimerized and/or aggregated proteins. In this case, the analysis was repeated four times, and the estimated purity was then expressed as an average of these measurements.

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