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Hypothesis

Co-operative binding of hsp60 may promote transfer hsp70 and correct folding of imported proteins in mitochondria

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I propose that a molecular chaperone hsp60 binds to and dissociates from the unfolded polypeptide or folding intermediate in a positively co-operative manner, but another chaperone hsp70 shows no such co-operativity. This could simply explain the fact that the protein newly imported in the mitochondrial matrix is transferred from hsp70 to hsp60 and hsp60 promotes correct folding of the substrate protein while hsp70 does not.

Molecular chaperone; Hsp70; Hsp60; Mitochondrion; Protein import

1. INTRODUCTION

In vitro denaturation/renaturation studies suggest that folding of a small protein is a self-assembly process in that all the information required is present in the amino acid sequences and it occurs spontaneously under the appropriate conditions [1]. However in cells, processes of protein folding may be modulated by a set of proteins referred to molecular chaperones or polypeptide chain binding proteins [2,3]. They include the members of the 70-kDa heat shock protein (hsp70) family and the 60-kDa heat shock protein (hsp60) family. Both hsp70 and hsp60 proteins bind preferentially to unfolded proteins or folding intermediates and release them in an ATP-dependent manner; single or multiple cycle(s) of hsp70 or hsp60 binding and release retard folding of the substrate polypeptide. The significance of the folding–modulating functions of the hsp70 and hsp60 proteins has been studied on translocation of precursor proteins across the mitochondrial membranes and their subsequent folding/assembly process in the matrix, the mitochondrial interior [4,5].

Most of the mitochondrial proteins are synthesized in the cytosol as a precursor protein with a presequence, an N-terminal peptide extension carrying a mitochondrial targeting signal. Many mitochondrial precursor proteins require cytosolic hsp70 to prevent aggregation or tight folding which abrogates transport competence [4]. They are released from cytosolic hsp70 on the mitochondrial surface and then inserted into and translocated across the outer and inner mitochondrial membranes in unfolded conformations. The unfolded polypeptide chain emerging in the matrix is trapped by mitochondrial hsp70 and the reverse translocation is likely prevented [4]. When mitochondrial hsp70 is mutated, transfer of precursors into mitochondria is defective and precursor proteins are found to be arrested in contact sites between the mitochondrial outer and inner membranes [6]. The newly imported protein in the matrix is transferred from hsp70 to mitochondrial hsp60 that promotes correct folding of the protein. The mutation in mitochondrial hsp60 results in the accumulation of unfolded mitochondrial proteins in the matrix [7].

Here two fundamental questions may arise. First, both hsp70 and hsp60 somehow retard protein folding, but why does hsp60 promote folding of the imported proteins while hsp70 does not? Second, why is the incoming unfolded polypeptide first bound to hsp70 and then transferred to hsp60 in the matrix? I propose here that co-operative binding of hsp60 to imported proteins could offer simple answers to these questions.

2. HSP60 MAY BIND TO AN UNFOLDED POLYPEPTIDE (OR FOLDING INTERMEDIATE) IN A CO-OPERATIVE MANNER

The hsp60 members including bacterial groEL protein and mitochondrial hsp60 form an oligomeric structure consisting of two stacked 7-mer rings [8]. In contrast, the hsp70 protein is not known to form such a large oligomeric structure [9]. The structural motif of the unfolded polypeptide to be recognized by hsp70 and hsp60 has not yet been clearly characterized, but such regions appear to be relatively small since the two members of the hsp70 family, BiP and cytosolic hsp70, could bind to peptides of as few as 10 amino acid residues [10] and groEL to that of 13 residues [11]. Thus, the unfold-

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ed protein imported newly into mitochondria may well offer multiple binding sites for the hsp70 protein or hsp60 as a 14-mer complex [3]; the molar ratio of binding of a 14-mer groEL to its substrate was indeed found to be one to one [12]. An approximate 1:1 stoichiometry was detected for binding of cytosolic hsp70 to a reduced and alkylated α-lactalbumin in vitro [9], but the complex formation in this case was unusually slow (the half-time ≅40 min) and hardly reflected a reaction taking place in cells.

Oligomeric proteins consisting of several polypeptide chains each of which carries a binding site for ligands often exhibit co-operativity in ligand binding, e.g. hemoglobin as a best-known example [1]. We thus assume that binding of oligomeric hsp60s to unfolded polypeptides is characterized by positive cooperativity while monomeric hsp70s do not show such cooperative binding. If a polypeptide chain P contains n binding sites for hsp70 (A) or hsp60 subunit (B) ($n \leq 14$), then the equilibrium among several states for the polypeptide (P)-hsp70 (A) or -hsp60 subunit (B) complexes can be considered as follows.

For hsp70,

$$P \underset{K}{\overset{A}{\rightleftharpoons}} PA \underset{K}{\overset{A}{\rightleftharpoons}} PA_2 \rightleftharpoons \cdots \rightleftharpoons PA_{n-1} \underset{K}{\overset{A}{\rightleftharpoons}} PA_n$$

Where K is a dissociation constant for hsp70 binding to the binding sites of the polypeptide chain. We do not consider any interactions among the binding sites within the substrate polypeptide.

For hsp60 subunits,

$$P \underset{K_1}{\overset{B}{\rightleftharpoons}} PB \underset{K_2}{\overset{B}{\rightleftharpoons}} PB_2 \rightleftharpoons \cdots \rightleftharpoons PB_{n-1} \underset{K_n}{\overset{B}{\rightleftharpoons}} PB_n$$

where K_1, K_2,..., K_n are the dissociation constants for PB, PB_2, ..., PB_n. Since only one polypeptide can bind to the hsp60 14-mer, B in the PB_n complex belongs to the same hsp60 oligomer. Positive cooperativity in the binding of hsp60 subunits and the polypeptide means $K_1 > K_2 > \ldots > K_n$.

Although the affinity of the free hsp60 oligomer for unfolded polypeptides may be lower than that of hsp70 ($K_1 > K$), binding of the polypeptide to one subunit of the hsp60 oligomer increases the affinities of the other subunit, leading to a higher stability of the final complexes of hsp60 and the substrate polypeptide than that of the hsp70-polypeptide complex ($K_n < K$). Indeed, positive co-operativity in groEL binding to unfolded proteins was indicated int he groEL-concentration dependence of the refolding of Rubisco (Fig. 3c of Ref. [13]) or retarding of the renaturation of the denatured dihydrofolate reductase (Fig. 1c of Ref. [12]).

3. CO-OPERATIVE RELEASE OF THE UNFOLDED PROTEIN FROM HSP60 CAN PROMOTE ITS SPONTANEOUS CORRECT FOLDING

Folding of an unfolded polypeptide chain is a co-operative and hierarchical process [14,15]. The unfolded protein first collapses within a millisecond into a compact folding intermediate containing several secondary structures (the molten globule state). Then the preformed secondary structure segments are organized into a specific tertiary structure in e.g. 0.1 s except for slow *cis–trans* isomerization of proline residues. This process requires concerted participation of long-range specific interactions that may cover the entire polypeptide chain. Recent evidence has shown that the groEL oligomer stabilizes the substrate protein in the molten globule state [12].

Binding of hsp60 to unfolded proteins with positive co-operativity means that the dissociation of the hsp60–intermediate complex is a cooperative process while dissociation ofthe hsp70–intermediate complex is not. Co-operative release of the folding intermediate from the hsp60 oligomer on ATP hydrolysis will make all the peptide segments available for long-range specific interactions that are indispensable for the correct organization of the secondary structure segments in the tertiary structure formation. In contrast, random and slow dissociation (turnover time of 5 min [10]) of the hsp70 molecules from the folding intermediate on ATP hydrolysis provides only limited availability of the correct partner segments for folding interactions. Thus incorrect long-range interactions of a limited number of secondary structure segments lead the polypeptide to incorrect folding pathways, resulting in a misfolded or unfolded state. Possible random re-binding of the dissociated hsp70 to the substrate protein may avoid the complete release of the hsp70 molecules from the protein and thereby prevent the unproductive and irreversible aggregation.

Of course even in the case of hsp60, formation of misfolded aggregates of the released protein must be avoided. This appears to be achieved by groES in the bacterial groEL-ES system; the groES oligomer consisting of 7 identical subunits likely keeps the released protein remaining in the central hole space of the groEL 14-mer and prevents its contact with the bulk solution [16]. Thus hsp60 mediates co-operative release and protection from the unfavorable intermolecular contact of the folding intermediate, which can guide the protein to its correct folding pathway. This model is different from that proposed by Martin et al., in which groES prevents the 'wholesale' dissociation of the substrate protein from groEL [12].

4. CO-OPERATIVITY REFLECTED IN THE ASSOCIATION RATE OF HSP60 MAY FACILITATE EFFECTIVE TRANSFER OF THE FOLDING INTERMEDIATE FROM HSP70

During or after translocation across the mitochondrial membranes, the unvolded precursor protein is transferred from hsp70 to hsp60 since precursor prote-

ins in transit through the mitochondrial membranes are found in a complex with hsp70 [17,18] while complexes of the imported protein and hsp60 are found only in the matrix [7].

We consider the competition between the proteins hsp60 and hsp70 for association with a binding site of the unfolded protein (P), assuming that the binding sites have partly overlapping specificity for hsp70 and hsp60; ternary complexes involving the substrate protein, hsp60 and hsp70 are neglected for simplicity. If the positive co-operativity in binding of P and hsp60 subunits (B) is reflected in not only the dissociation constants but also the association rate constants, $k_1, k_2,...k_n$, where $k_1, k_2,...k_n$ are the rate constants for the association of B with P, $PB,...,PB_{n-1}$, respectively, k_1 should be smaller than $k(k_1<k)$, k being the rate constant for the association of hsp70 (A) with P, $PA,...,PA_{n-1}$.

If $k_1<<k$, proportion of the complexes involving hsp60 subunits (B) and hsp70 (A) will reflect their relative association rates in the time range long enough for complex formation but not long enough for significant displacement of hsp60 and hsp70 between the complexes [19].

$$\frac{k_1}{k} = \frac{\ln ([B_0]/[B_{free}])}{\ln ([A_0]/[A_{free}])}$$

where $[A_0]$ and $B_0]$ are the initial concentrations of hsp70 and hsp60 subunits, $[A_{free}]$ and $[B_{free}]$ the concentrations of free hsp70 molecules and free hsp60 subunits, respectively. Since $k_1<k$, the folding intermediate P associates with the hsp70 protein (A) preferentially over the hsp60 (B) oligomer in the short time range (within a minute). It should be noted that the translocation process of unfolded polypeptide chains across the mitochondrial membranes falls in this time range [3].

In the longer time range, the proportion of the complexes PA_n and PB_n will relax to their equilibrium values:

$$\frac{K_1}{K} \cdot \frac{K_2}{K} \cdots \frac{K_n}{K} = \frac{[PA_n]}{[A_{free}]^n} \cdot \frac{[B_{free}]^n}{[PB_n]}$$

If the cooperativity in binding of polypeptides and hsp60 is high enough to make $(K_1/K)(K_2/K)...(K_n/K)<1$ and the mitochondrial matrix contains comparable and excess amounts of hsp70 and hsp60 ($[B_{free}]/[A_{free}]\cong 1$), then $[PB_n]>[PA_n]$ at equilibrium. Thus the unfolded protein P will be transferred from hsp70 (A) to hsp60 (B) in a rather long time range (in the order of say 10 min); then acquisition of the correct tertiary (and oligomeric) structure of the protein will follow in a proper time range.

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