Comparison of Folding Rates of Homologous Prokaryotic and Eukaryotic Proteins*

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The rate of polypeptide chain elongation is up to one order of magnitude faster in prokaryotic cells than in eukaryotes. Here we report that the rates of in vitro refolding of orthologous prokaryotic and eukaryotic proteins correlate with their differential rates of biosynthesis. The mitochondrial and cytosolic aspartate aminotransferases of chicken and aspartate aminotransferase of Escherichia coli show pairwise sequence identities of 41–48% and nearly identical three-dimensional structures. Nevertheless, the prokaryotic enzyme refolded 6 times faster (at 25 °C) than the eukaryotic isoenzymes after denaturation in 6 M guanidine hydrochloride. Prokaryotic malate dehydrogenase and lactate dehydrogenase also renatured faster than their orthologous eukaryotic counterparts, suggesting that evolutionary pressure has adapted the rate of folding to the rate of elongation of polypeptide chains.

The rate of polypeptide chain elongation in prokaryotes (1, 2) is 4–10 times faster than in eukaryotic cells (3, 4). Here, we address the question of whether the faster rate of protein synthesis in prokaryotes correlates with faster rates of protein folding. The ultimate determinant of the folding rate of proteins is the primary structure including proline content. However, other factors such as protein size, chain topology, and thermodynamic stability are thought to contribute to a wide range of kinetic patterns (5–7). For our study, we chose three sets of homologous (or more precisely, orthologous) eukaryotic and prokaryotic proteins (Table I) to eliminate differences in size or chain topology. The AspATs† of chicken (mitochondrial and cytosolic isoenzymes) and of Escherichia coli possess nearly identical structures (8). All three enzyme variants are $\alpha_2$ dimers, each subunit composed of 13 $\alpha$-helices, a 7-stranded $\beta$-sheet core, and one molecule of the coenzyme PLP. The two other sets of orthologous proteins that we examined were prokaryotic and eukaryotic MDH and LDH. MDH is an $\alpha_2$-dimer with 8 $\alpha$-helices and 5–6 $\beta$-strands/subunit, and LDH is a tetramer with 9 $\alpha$-helices and 6–10 $\beta$-strands/subunit.

EXPERIMENTAL PROCEDURES

Proteins—Mitochondrial and cytosolic AspAT were purified from chicken heart as described previously (9). E. coli AspAT was overproduced in strain TY103 harboring pKDHE 19/AspAT (kindly provided by Dr. H. Kagamiyama, Osaka Medical College) and purified according to published protocols (10). Prior to experimentation, all AspATs were subjected to Sephadex G-25 gel filtration (Amersham Pharmacia Biotech) to remove excess cofactor PLP. The concentrations of the purified enzymes in the PLP form were determined photometrically ($e_{280}$ = 4.7$ \times 10^4$ M$^{-1}$ cm$^{-1}$ for E. coli AspAT and $e_{340}$ = 7.0$ \times 10^4$ M$^{-1}$ cm$^{-1}$ for both mitochondrial and cytosolic AspATs; Ref. 9). Porcine mitochondrial, cytosolic aspartate and E. coli MDH, as well as chicken muscle (type XXXIV), chicken heart (type VIII), and Bacillus stearothermophilus LDH, were purchased from Sigma.

Denaturation and Refolding—Denaturation of AspAT was verified by determining the number of sulfhydryl groups reactive with 1 mM 5,5’-dithio-bis(2-nitrobenzoic acid). For all proteins tested, CD spectrophotometry confirmed the complete loss of secondary structure. Renaturation of AspAT was initiated by a 350-fold dilution of the solution of the denatured enzyme in 6 M GdnHCl with refolding buffer (25 mM Hepes, 100 mM KCl, 1 mM EDTA, pH 7.0) in Minisorb tubes (Nunc). The concentrations of the proteins in the refolding solution were 3 or 6 $\mu$g/ml, corresponding to subunit concentrations of 67 and 134 mM if the refolding was followed by enzymic activity or fluorescence, respectively. A glass-coated magnetic stirrer was used in the former case, and manual mixing was applied in the latter case. Refolding of MDH was initiated by a 390-fold dilution to a final subunit concentration of 85 mM with refolding buffer (100 mM potassium phosphate, 1 mM 1,4-dithio-bis-m-tol, 1 mM EDTA, pH 7.4). Refolding of LDH was initiated by a 210–550-fold dilution to a final subunit concentration of 85 mM with refolding buffer (100 mM Tris-HCl, 1 mM 1,4-dithio-bis-m-tol, 1 mM EDTA, pH 7.5).

Measurement of Enzymic Activity—A sample of the refolding solution was transferred into the activity assay at the indicated times. The ensuing dilution was 8-fold. The activity of the refolded protein was calculated from the initial linear segment of the reaction progress curve. AspAT activity was determined in a coupled assay with 1.8 units of MDH and 200 $\mu$M NADH in 50 mM sodium phosphate buffer, 20 mM aspartate, 20 mM 2-oxoglutarate, pH 7.5, at 25 °C. MDH activity was measured with freshly dissolved 20 mM oxalacetate and 200 $\mu$M NADH in 100 mM potassium phosphate, pH 7.4, at 25 °C. LDH activity was measured with 30 mM pyruvate and 200 $\mu$M NADH in 200 mM Tris HCl, pH 7.3, at 37 °C. GdnHCl at the low concentrations present (1–4 mM) did not interfere with the activity measurements.

Fluorescence Measurements—The intrinsic fluorescence of AspAT (132 nM subunit concentration) was recorded in a 1×1-cm cuvette with a Spex Fluorolog spectrophotofluorimeter. For kinetic refolding experiments, the wavelengths of excitation and emission were set at 290 nm (bandwidths 1.8/1.8 nm) and 360 nm (9.2/18.5 nm), respectively.

RESULTS AND DISCUSSION

The rates of refolding of the prokaryotic proteins after denaturation in 6 M guanidine hydrochloride (GdnHCl) for 40 min proved with exception faster than those of their orthologous eukaryotic counterparts (Fig. 1), the ratios between the folding rates of prokaryotic and eukaryotic enzymes at 25 °C varying from 1.6 to 82 (Table II). Lowering the temperature increased the difference between the rates of folding of prokaryotic and eukaryotic AspATs. At 2 °C, reactivation of prokaryotic AspAT was 20 times faster than the refolding of the eukaryotic enzyme; at 37 °C, the physiological temperature, the prokaryotic enzyme still folded 5 times faster. The rate of refolding of prokaryotic AspAT was 20 times faster than the refolding of their orthologous eukaryotic counterparts, suggesting that evolutionary pressure has adapted the rate of folding to the rate of elongation of polypeptide chains.
Folding of Homologous Prokaryotic and Eukaryotic Proteins

Amino acid sequence identities of the investigated sets of orthologous prokaryotic and eukaryotic proteins

The degree of identity was determined with the program SimAli (P. K. Mehta and J. Heringa, unpublished). In all cases, homology has been confirmed by similarity in three-dimensional structure (Protein Data Bank accession numbers: AspAT: 1asl, 2cst, 1tat; MDH: 1emd, 4mdh,1mld; LDH: 1ldb, 5ldh, 9ldt; the three-dimensional structure of LDH from pig rather than chicken is known).

| Protein                          | Compared sequences                              | Sequence identity % |
|----------------------------------|------------------------------------------------|---------------------|
| Aspartate aminotransferase       | E. coli/chicken mitochondrial                   | 41                  |
|                                  | E. coli/chicken cytosolic                      | 41                  |
|                                  | Chicken cytosolic/mitochondrial                | 48                  |
| Malate dehydrogenase             | E. coli/pig mitochondrial                      | 59                  |
|                                  | E. coli/pig cytosolic                          | 23                  |
|                                  | Porcine cytosolic/mitochondrial                | 23                  |
| Lactate dehydrogenase            | B. stearothermophilus/chicken heart            | 74                  |
|                                  | B. stearothermophilus/chicken muscle           | 36                  |
|                                  | Chicken heart/muscle                           | 36                  |

Comparison of the refolding rates of prokaryotic and eukaryotic proteins

The rate constants (k_{obs} for the first rapid phase) were calculated from the data given in Fig. 1 and additional experiments of the same kind.

| Protein pair                        | Ratio of k_{obs} values |
|-------------------------------------|-------------------------|
|                                    | 2°C 15°C 25°C 37°C      |
| E. coli AspAT vs. cytosolic AspAT   | 20.2 7.7 6.2 4.9        |
| E. coli AspAT vs. mitochondrial AspAT | 25 7.7 6.0             |
| E. coli MDH vs. cytosolic MDH       | 11                      |
| E. coli MDH vs. mitochondrial MDH   | 82                      |
| B. stearothermophilus LDH vs. muscle LDH | 8                |
| B. stearothermophilus LDH vs. heart LDH | 1.6              |

The close structural similarity of the proteins compared within a given set of homologs (Table I) allows the conclusion that the primary structure rather than the length of the polypeptide chain or particular features of the native spatial structure determines the specific refolding behavior. All three AspATs possess two conserved cis-peptidyl proline bonds (Pro-138 and Pro-195; the numbering corresponds to that of pig cytosolic AspAT) among a total of 15 to 20 proline residues (13–16). The two conserved cis proline residues lie in close spatial proximity to one another on the surface of the large, coenzyme-binding domain. In E. coli AspAT, the two conserved cis proline residues have recently been substituted with alanine. Neither mutation brought about significant changes in three-dimensional structure, thermodynamic stability, and enzymatic activity (16). Apparently, the invariance of these two cis peptidyl proline bonds is not due to structural or functional reasons. The changes in folding rates of the mutant enzymes suggest, however, that these cis proline residues are located at positions within the polypeptide chain where their isomerization might control the rate of folding (6, 17). Does trans-cis proline isomerization underlie the observed difference in re-
and activation enthalpies (−9 kcal mol⁻¹) of all three AspATs were nearly identical. Apparently, Xaa-Pro isomerization is the rate-limiting step in the refolding of cytosolic and mitochondrial AspAT after long-term denaturation. This interpretation seems plausible although both human cyclophilin A and peptidyl-proline cis-trans isomerase of E. coli, added in a 20-fold molar excess, failed to accelerate the reactivation of cytosolic AspAT after a 40-min denaturation. Presumably, steric reasons prevented the enzymes from catalyzing the trans-cis isomerization. In E. coli AspAT, in which peptidyl-proline isomerization is of course also an integral part of the folding process, the isomerization is apparently facilitated by specific effects of side chains adjacent in sequence or space (6, 25).

CONCLUSION

Are data on in vitro refolding relevant for in vivo situations? In the cell, the folding of most proteins is probably a cotranslational process or, in the case of certain proteins, a cotranslational process (26). However, the rates of the consecutive steps in biosynthetic folding may be assumed to correlate with the rates of the corresponding steps in the refolding of complete polypeptide chains. The present data allow the following conclusions. 1) Homologous proteins with virtually superimposable three-dimensional structures may markedly differ in their rate of folding. 2) Prokaryotic representatives of a given protein fold faster than their orthologous eukaryotic counterparts. Faster folding in prokaryotes in which chain elongation is faster minimizes the occurrence of unfolded nascent proteins. Conceivably, part of the difficulties encountered in expressing eukaryotic proteins in prokaryotic cells might be due to a combination of a fast rate of synthesis and a slow rate of folding, which favors aggregation and proteolytic degradation. 3) In the case of prokaryotic and eukaryotic AspATs, the rate of trans-cis proline isomerization appears to have been modulated to control the rate of folding, indicating yet another regulatory role (27, 28) of peptidyl-proline isomerization.

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FIG. 2. Effect of duration of exposure to 6 M GdnHCl on the kinetics of refolding of AspATs. The enzymes were denatured in 6 M GdnHCl for 40 min (●, －－－) or 40 s (○, －－－). The 40-s pulse in 6 M GdnHCl at 25 °C was sufficient for complete denaturation, as indicated by the CD spectra and measurements of catalytic activity. The CD minimum at 220 nm of the native proteins disappeared completely in 6 M GdnHCl, with denaturation occurring within 20 s, the dead time of handling. The temperature for refolding was 25 °C in the case of E. coli and cytosolic AspAT and 15 °C in the case of mitochondrial AspAT. Measurements with mitochondrial AspAT at 25 °C proved to be imprécise because of the low yield. The refolding was followed by enzymic activity and intrinsic fluorescence. Invariably, renaturation resulted in a blue shift of the emission maximum and a decrease in intrinsic fluorescence intensity, which are due to exposure of the tryptophan residues to a less polar environment (21). The protein concentration was 68 and 132 nM for the fluorescence and activity measurements, respectively. For details, see "Experimental Procedures."
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