Differential effects of molecular chaperones on refolding of homologous proteins

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Abstract Three homologous aspartate aminotransferases with virtually identical spatial structures and pairwise amino acid sequence identities of >40% differ markedly with respect to the yield of renaturation upon dilution from 6 M guanidine hydrochloride (mitochondrial << cytosolic < *Escherichia coli*). The enzymes also respond differently to molecular chaperones. GroEL/GroES, the Hsp60 homolog of *E. coli*, increased considerably the yield of renaturation of mitochondrial aspartate aminotransferase and to a lesser extent that of its cytosolic counterpart, but not that of the *E. coli* enzyme. DnaK/DnaJ/GrpE, the Hsp70 system of *E. coli*, also increased the yield of renaturation of mitochondrial aspartate aminotransferase. Apparently, specific features in the amino acid sequence or the folding pathway which are independent of the final secondary and tertiary structure determine the interactions of the folding proteins with the chaperone systems.

Key words: Protein folding; Hsp70; Hsp60; Molecular chaperone; Aspartate aminotransferase

1. Introduction

Molecular chaperones do not convey information for the folding of a protein but guide the nascent polypeptide along the folding and assembly pathway. They increase the efficiency of folding by decreasing off-pathway interactions that lead to aggregation (for a recent review, see [1]). The most extensively investigated chaperone families are the Hsp60 and Hsp70 proteins. The Hsp60 proteins are large double-ring complexes that bind polypeptide chains in their central cavity and mediate folding by multiple rounds of ATP-dependent binding and release [2]. Among other functions, they assist the folding and assembly of imported proteins in mitochondria [3]. Chaperones of the Hsp70 type are ubiquitous and have been implicated in the stabilization of unfolded polypeptides before their assembly or translocation into organelles, e.g. mitochondria, as well as in the stabilization of newly translocated polypeptides prior to their folding and assembly [4,5]. DnaK, the Hsp70 homolog of *Escherichia coli*, depends in its action on the accessory chaperones DnaJ and GrpE. The kinetics of binding and the affinity for ligands are modulated by binding and hydrolysis of ATP [6,7].

Proteins with diverging biogenetic pathways and dissimilar intracellular locations may be expected to differ in their folding modes and interactions with chaperone systems. To test this proposition, three homologous proteins, mitochondrial, cytosolic and *E. coli* aspartate aminotransferase, were denatured *in vitro* and compared with respect to their yield of renaturation in the presence and absence of chaperones, i.e. DnaK/DnaJ/GrpE and GroEL/GroES. The three target proteins are α_2 dimers with virtually superimposable tertiary structures and a pairwise amino acid sequence identity of 40–47%. The mitochondrial isoenzyme is synthesized as a precursor with an aminoterminal leader sequence and is posttranslationally imported into mitochondria [8]. The three homologous proteins were found to differ markedly both in the yield of renaturation and in the response to the chaperone systems.

2. Materials and methods

2.1. Protein purification

Mitochondrial and cytosolic aspartate aminotransferase (AspAT) were purified from chicken hearts as described previously [9]. *E. coli* aspartate aminotransferase was overproduced in the *E. coli* TY103 strain harboring pKDHE 19/AspAT (kindly provided by Dr. H. Kagamiyama) and purified according to published protocols [10,11]. DnaK was overexpressed in the *E. coli* JM83 strain harboring pTTQ19 (a gift from Dr. C. Georgopoulos) and purified as previously described [6]. DnaJ, GrpE, GroEL and GroES were generously provided by Dr. H.-J. Schönfeld and S. Axmann.

2.2. Determination of protein concentration and aminotransferase activity

The concentration of the purified enzymes in the pyridoxal-5′-phosphate form was determined photometrically at 280 nm with the absorption coefficients $\varepsilon = 4.7 \cdot 10^4$ $M^{-1} \cdot cm^{-1}$ for *E. coli* AspAT [12] and $\varepsilon = 7.0 \cdot 10^4$ $M^{-1} \cdot cm^{-1}$ for both mitochondrial and cytosolic AspATs [13]. Aminotransferase activity was measured in a coupled assay with malate dehydrogenase in 50 mM sodium phosphate, 20 mM aspartate, 20 mM 2-oxoglutarate, pH 7.5, at 25°C. Guanidine hydrochloride at the low concentrations present after dilution in the refolding experiments did not disturb the activity measurements.

2.3. Denaturation of aspartate aminotransferases

The AspATs were denatured in 6 M guanidine hydrochloride (Fluka, MicroSelect), 50 mM sodium phosphate, pH 7.5, for 1 to 2 h at 25°C. The denaturation was verified by determination of the number of sulfhydryl groups reactive with 1 mM 5,5′-dithio-bis-(2-nitro-benzoic acid) (Fluka) [14]. In the denatured state, all 5 sulfhydryl groups of mitochondrial and *E. coli* AspAT as well as all 4 sulfhydryl groups of cytosolic AspAT were found to react.

2.4. Refolding experiments

Renaturation was started by dilution of the denatured enzyme 250–480 fold to a final subunit concentration of 67 nM with refolding buffer containing 25 mM Hepes-NaOH, 100 mM KCl, 1 mM 1,4-dithio-DL-threitol, 0.1 mM pyridoxal-5′-phosphate, 0.1% (w/v) ovalbumin, 1 mM ATP, 5 mM magnesium chloride, pH 7.0, in Minisorb tubes (Nunc) at 0, 25 or 37°C. The molar ratio of AspAT/DnaK/DnaJ/GrpE/GroEL/GroES was 1:5:2:5:1:1. The yield of reactivation was followed as a function of time. The maximum yield was reached after approximately 1 h for renaturation at 25°C and 37°C, and after several days at 0°C.

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Abbreviations: AspAT, aspartate aminotransferase; Hsp60, heat shock protein 60; Hsp70, heat shock protein 70.

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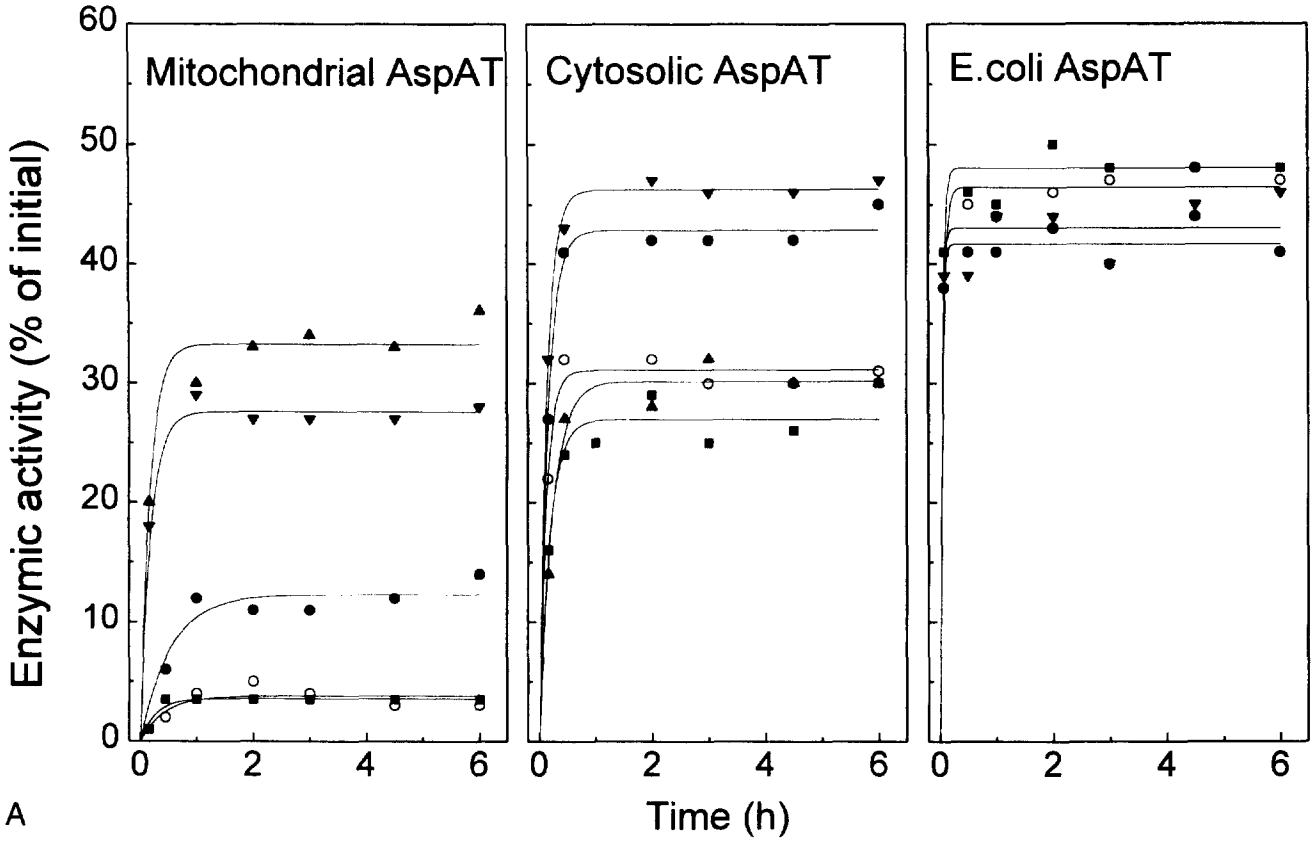
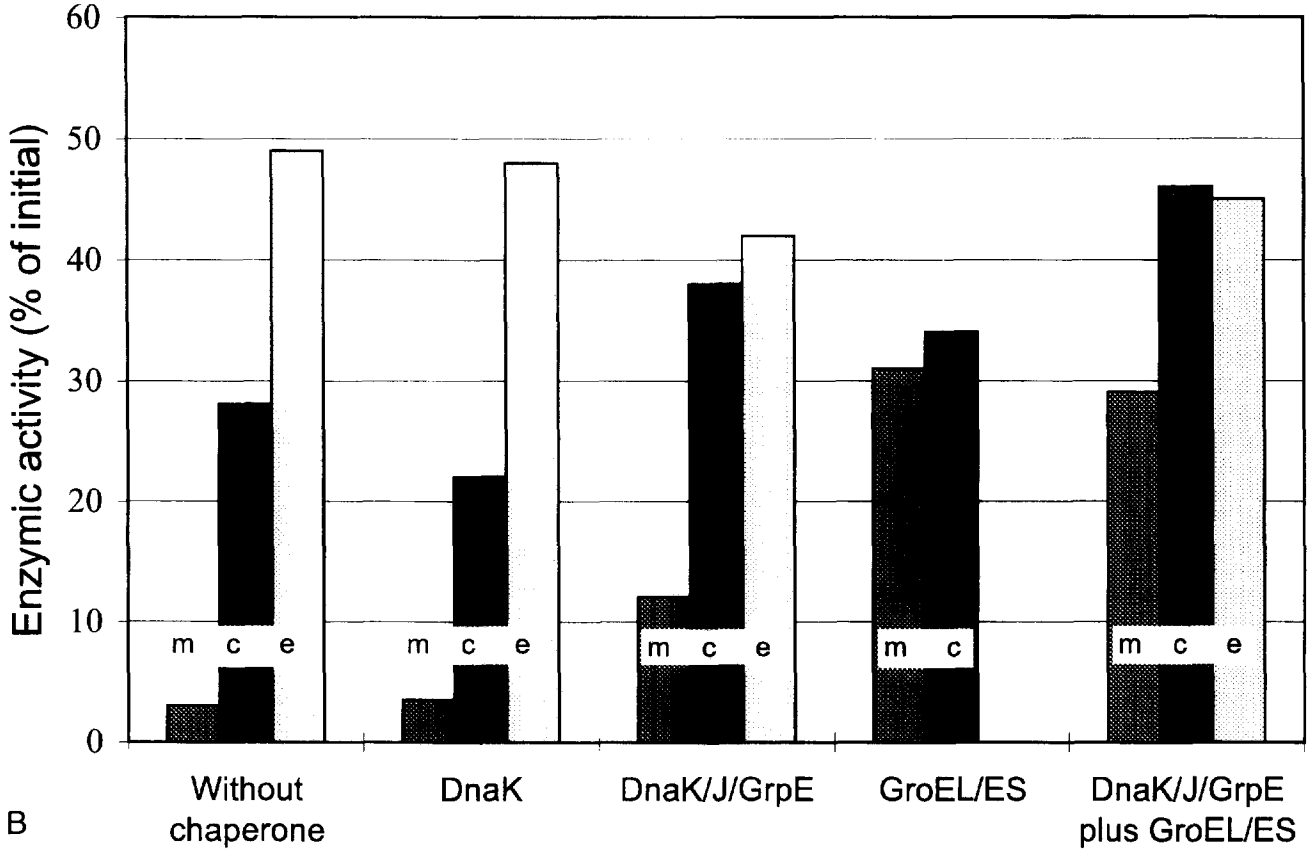

Mitochondrial AspAT
Cytosolic AspAT
E.coli AspAT
Enzymic activity (% of initial)
Time (h)
0
10
20
30
40
50
60
0
2
4
6

A


Enzymic activity (% of initial)
0
10
20
30
40
50
60
m c e
m c e
m c e
m c
m c e
Without chaperone
DnaK
DnaK/J/GrpE
GroEL/ES
DnaK/J/GrpE plus GroEL/ES

B

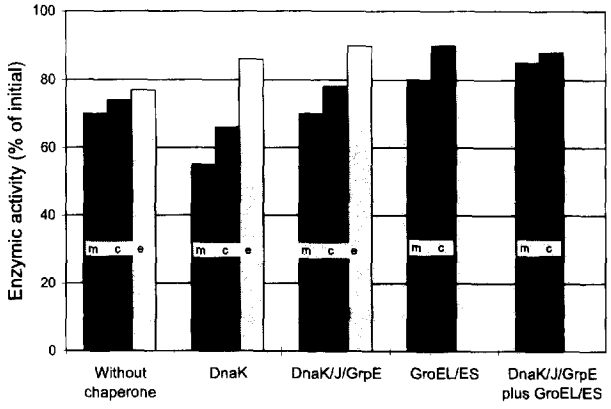


Fig. 2. Maximum yield of renaturation of the three AspATs in the presence and absence of chaperones at 0°C. Denotations and conditions are the same as in Fig. 1B.

3. Results

3.1. Renaturation of AspAT variants in the absence of chaperones

The yields of spontaneous reactivation of the three AspATs differ significantly. Under identical conditions for refolding at 25°C, mitochondrial AspAT was reactivated to only 3%, whereas cytosolic AspAT was reactivated to 28% and *E. coli* AspAT to 48% of the initial enzymic activity (Fig. 1). The higher the yield of reactivation, the higher was the rate of the reactivation (Fig. 1A). If renaturation was carried out at 0°C, the yield was similar (70–77%) for all three enzymes (Fig. 2). At 37°C, only 3% for mitochondrial AspAT, 6% for cytosolic AspAT and 16% for *E. coli* AspAT were measured.

3.2. Differential influence of Hsp70 proteins on renaturation of AspAT variants

Refolding experiments carried out in the presence of a 5-fold molar excess of DnaK over AspAT showed no significant difference in comparison to refolding in the absence of the chaperone (Figs. 1 and 2). However, if the experiments were carried out in the presence of the complete Hsp70 system of *E. coli* consisting of DnaK/DnaJ/GrpE, the reactivation yield of mitochondrial AspAT at 25°C was four times higher than without chaperones. Thus, as reported previously [15,16], the interaction of DnaK with the cochaperones DnaJ and GrpE is essential for the effect of DnaK. If DnaK was substituted with mitochondrial Hsp70 of yeast (kindly provided by Dr. M. Horst), no further increase in the yield of refolding of mAspAT was observed. This result confirms the notion [17,18] that the cochaperones of the Hsp70 system act only with specific Hsp70 homologs. The refolding of cytosolic AspAT was furthered to a lesser extent and that of *E. coli* AspAT was scarcely influenced. At 0°C, the effect of the Hsp70 proteins was less marked than at 25°C, the yield of unassisted renaturation being already in the range of 70–77%. At 37°C, the chaperones did not increase the generally low yield of renaturation.

3.3. Hsp60 system markedly enhances the yield of refolding of mitochondrial AspAT

The reactivation yield of mitochondrial AspAT was increased about 10 fold in the presence of GroEL/GroES at 25°C (Fig. 1). The rate of the process was also increased. Neither GroEL nor GroES alone influenced the reactivation of mitochondrial AspAT. The yield of reactivation of cytosolic AspAT was increased only about 1.3fold. If both chaperone systems, the Hsp60 plus Hsp70 proteins, were present in the reaction mixture, no further significant increase of the reactivation yields was observed. The reactivation of *E. coli* AspAT was not affected. At 0°C, neither chaperone system further increased the already high yield of reactivation (Fig. 2). GroEL/GroES even delayed refolding of mitochondrial AspAT at 0°C (not shown). At 37°C, the chaperonins were without effect.

4. Discussion

The results of this study show that (1) the yield of spontaneous refolding is highest for *E. coli* AspAT and lowest for the mitochondrial enzyme; (2) both systems, DnaK/DnaJ/GrpE and GroEL/GroES, are more effective in increasing the yield of reactivation of the mitochondrial than of the cytosolic isoenzyme; and (3) the effect of the Hsp60 system is significantly greater than that of the Hsp70 system for the mitochondrial enzyme. The markedly higher yield of spontaneous refolding of all three AspATs at 0°C supports the notion that incomplete refolding at higher temperatures is largely due to off-pathway aggregation.

The two intracellularly heterotopic isoenzymes of AspAT are both coded for by nuclear DNA and are synthesized on free cytosolic ribosomes [8]. Their posttranslational fates, however, are quite different. The cytosolic protein may be assumed to start folding during translation and is not subject to any processing. The mitochondrial protein is synthesized as a precursor with an aminoterminal presequence [19] and is thought to be kept in an unfolded state probably by interaction with cytosolic Hsp70. Mitochondrial AspAT has indeed been found to bind the coenzyme only after translocation into the mitochondria [20]. Translocation through the mitochondrial membrane presumably is assisted by mitochondrial Hsp70. After splitting off the presequence by a leader peptidase, the enzyme is folded under assistance of the mitochondrial Hsp70 and Hsp60 systems (for a review on mitochondrial protein import, see [21]). The findings of the present study agree with and extend previous studies on the spontaneous and GroEL/ES-assisted folding of AspAT [22] and malate dehydrogenase isoenzymes [23]. The lower yield of spontaneous refolding of mitochondrial AspAT as compared to cytosolic AspAT might reflect a slower cotrans-

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Fig. 1. Renaturation of the three AspATs in the presence and absence of chaperones at 25°C. (A) The enzymes had been denatured in 6 M guanidine hydrochloride; renaturation was followed after dilution with folding buffer. The folding buffer with or without the chaperones contained ATP and was temperature-equilibrated before adding the denatured enzymes. For experimental details, see section 2. ○, Reactivation without chaperones; ■, in the presence of DnaK; ●, in the presence of DnaK/DnaJ/GrpE; ▲, in the presence of GroEL/ES; ▼, in presence of DnaK/DnaJ/GrpE plus GroEL/ES. (B) The maximum yield of reactivation was determined after several hours, when no further increase was observed. m, mitochondrial aspartate aminotransferase; c, cytosolic aspartate aminotransferase; e, *E. coli* aspartate aminotransferase.

lational folding of this isoenzyme or a higher affinity for chaperones combined with a higher propensity to form intermolecular aggregates in their absence. A higher affinity for chaperones might underlie their greater effect on the renaturation yield of mitochondrial AspAT. Hence, the low yield of spontaneous refolding and the high responsiveness to chaperone action of the mitochondrial isoenzyme might relate to the posttranslational uptake of its precursor into mitochondria. Conceivably, the differences in the folding behavior of the mitochondrial and cytosolic isoenzymes might have been even larger if the precursor of mitochondrial AspAT, the presequence of which has been found to be bound tightly to DnaK [6,24], and the eucaryotic and mitochondrial Hsp60 and Hsp70 homologs had been used. Remarkably, *E. coli* AspAT shows the highest yield in refolding at all temperatures. This might relate to the fact that translation in prokaryotes is faster by one order of magnitude than in eukaryotes [25] necessitating faster folding which reduces the chance of unproductive off-pathway interactions. In this context, the observation that GroEL/ES not only increases the yield but also the rate of reactivation of mitochondrial AspAT is of particular interest as it suggests a catalytic effect of the chaperone on protein folding and assembly which seems to deserve further experimental attention.

The three homologous AspATs have a virtually superimposable three-dimensional structure. We may conclude that the differences both in spontaneous folding and in the interaction with the Hsp70 chaperones are solely due to differences in their primary structure and independent of the final folding pattern of the polypeptide chain. In the case of the Hsp60 chaperones, differences in the structures of folding intermediates of the three AspATs might be important. Previous experiments on the rate of import into mitochondria of chimeric preproteins have shown that the mature part of the precursor of mitochondrial AspAT contains structural features essential for rapid importation [26]. The cytosolic isoenzyme with the presequence of the precursor attached to it was four times more slowly imported than the authentic precusor. The present study suggests that in addition to positive charge [27], the mode and rate of folding and the affinity for molecular chaperones might be features of the mature part of imported mitochondrial proteins determining their rate of importation. Studies under way in our laboratory attempt to identify these features.

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References

[1] Becker, J. and Craig, E.A. (1994) Eur. J. Biochem. 219, 11–23.
[2] Hartl, F.U. (1994) Nature 371, 557–559.
[3] Hartl, F.U., Martin, J. and Neupert, N. (1992) Annu. Rev. Biophys. Biomol. Struct. 21, 293–322.
[4] Schatz, G. (1993) Protein Science 2, 141–146.
[5] Hartl, F.U. and Martin, J. (1995) Current Opin. Struct. Biol. 5, 92–102.
[6] Schmid, D., Baici, A., Gehring, H. and Christen, P. (1994) Science 263, 971–973.
[7] McCarty, J.S., Buchberger, A., Reinstein, J. and Bukau, B. (1995) J. Mol. Biol. 249, 126–137.
[8] Sonderegger, P., Jaussi, R., Christen, P. and Gehring, H. (1982) J. Biol. Chem. 257, 3339–3345.
[9] Gehring, H., Christen, P., Eichele, G., Glor, M., Jansonius, J.N., Reimer, A.-S., Smit, J.D.G. and Thaller, C. (1977) J. Mol. Biol. 115, 97–101.
[10] Herold, M. and Kirschner, K. (1990) Biochemistry 29, 1907–1913.
[11] Vacca, R.A., Christen, P., Malashkevich, V.N., Jansonius, J.N. and Sandmeier, E. (1995) Eur. J. Biochem. 227, 481–487.
[12] Kuramitsu, S., Hiromi, K., Hayashi, H., Morino, Y. and Kagamiyama, H. (1990) Biochemistry 29, 5469–5476.
[13] Gehring, H. and Christen, P. (1978) J. Biol. Chem. 253, 3158–3163.
[14] Riddles, P.W., Blakeley, R.L. and Zerner, B. (1979) Anal. Biochem. 94, 75–81.
[15] Buchberger, A., Schröder, H., Büttner, M., Valencia, A. and Bukau, B. (1994) Nature Struct. Biol. 1, 95–101.
[16] Wall, D., Zylicz, M. and Georgopoulos, C. (1995) J. Biol. Chem. 270, 2139–2144.
[17] Schlenstedt, G., Harris, S., Risse, B., Roland, L. and Silver, P.A. (1995) J. Cell Biol. 129, 979–988.
[18] Cyr, D.M. (1995) FEBS Lett. 359, 129–132.
[19] Jaussi, R., Cotton, B., Juretic, N., Christen, P. and Schümperli, D. (1985) J. Biol. Chem. 260, 16060–16063.
[20] Sharma, C.P. and Gehring, H., (1986) J. Biol. Chem. 261, 11146–11149.
[21] Stuart, R.A., Cyr, D.M., Craig, E.A. and Neupert, W. (1994) Trends Biol. Sci. 19, 87–92.
[22] Mattingly, J.R., Iriarte, A. and Martinez-Carrion, M. (1995) J. Biol. Chem. 270, 1138–1148.
[23] Staniforth, R.A., Cortès, A., Burston, S.G., Atkinson, T., Holbrook, J.J. and Clarke, A.R. (1994) FEBS Lett. 344, 129–135.
[24] Schmid, D., Jaussi, R. and Christen, P. (1992) Eur. J. Biochem. 208, 699–704.
[25] Wolfe, S.L., Molecular and cellular biology, Wadsworth Publishing Company, (1993) p. 660.
[26] Hartmann, C.M., Lindenmann, J.-M., Christen, P. and Jaussi, R. (1991) Biochem. Biophys. Res. Commun. 174, 1232–1238.
[27] Hartmann, C., Christen, P. and Jaussi, R. (1991) Nature 352, 762–763.