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Accelerated Protein Aggregation Induced by Macrophage Migration Inhibitory Factor under Heat Stress Conditions

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Abstract—Kinetics of thermal aggregation of model protein substrates (glycogen phosphorylase *b* from rabbit skeletal muscle and yeast alcohol dehydrogenase) were investigated under heat stress conditions (41-48°C) in the presence of macrophage migration inhibitory factor (MIF), a heat-stable hydrophobic protein (12.5 kD). Anti-chaperone MIF activity found by turbidimetry manifests itself in significantly accelerated protein aggregation and increased limiting value of apparent optical absorption at 360 nm and $t \to \infty$ in the sub-stoichiometric range of MIF concentrations. The aggregation kinetics is shown to have cooperative character. Possible reversibility of aggregation after removal of denaturing conditions was demonstrated using alcohol dehydrogenase aggregation at a temperature close to the physiological level (41.5°C). This reversibility is caused by solubility of aggregates and stabilization of oligomeric structure of the substrate as a result of MIF binding to the partially denatured protein. The data suggest that in spite of distinct anti-chaperone effect, the chaperone-like activity of MIF can be observed in the case of heat stress removal and restoration of the system to normal conditions.

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Molecular chaperones can protect proteins, which have lost native conformation under various stress conditions. This capacity manifests itself in interaction of chaperones with hydrophobic sites of unfolded or improperly folded proteins; this interaction prevents mutual binding of these proteins and further aggregation and finally results in conservation of their native state. Chaperones participate in various intracellular processes including co-translational folding of polypeptide chains on their biosynthesis, protein translocation through membranes, and inclusion of individual proteins into supramolecular structures [1-3].

Activation of synthesis of molecular chaperones known as heat shock proteins (HSP) is observed in cells of all organisms at temperatures above the physiological level. Some of these chaperones (HSP70, GroEL) provide refolding of proteins subjected to heat damage, to native state, whereas others (α-crystallin, small HSP) inhibit aggregation and stabilize protein structure but do not participate in their refolding [4-8]. Protein aggregates may be solubilized and correctly refolded resulting in formation of biologically active conformation by the action of a system of ATP-dependent chaperones and co-chaperones—constituents of a multichaperone network. Folding enzymes (peptidyl-prolyl-*cis-trans*-isomerase and protein disulfide isomerase) and various chaperone-like proteins are often involved in this network [4, 5, 9-13].

It is of great interest to study the mechanisms of action of molecular chaperones not only because of the problems caused by the aggregation processes while producing recombinant proteins, but also by the necessity of developing efficient ways for cell protection in pathological states as a result of destabilization of protein conformation.

Studies on chaperones are intriguing because of their variety and broad action spectrum in cell compartments, and the number of discovered chaperone-like proteins is constantly increasing. However, despite multiple experiments aimed at the study of the protective role of chaperones under stress conditions, the molecular mechanisms of their action remain unclear.

In many cases, *in vitro* studies are complicated by the fact that the effect of chaperones significantly depends on experimental conditions (characteristics of protein substrate and chaperone, stoichiometric ratios, temperature,

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buffer, form and dimensions of aggregates, their solubility, etc.). Change in these conditions can drastically change the capacity of chaperone for preventing aggregation of protein substrates [14]. Under certain conditions, so-called anti-chaperone activity—unexpected chaperone capacity for accelerating protein aggregation under stress conditions—is observed [15].

All chaperones now known possess some general properties: they have hydrophobic domains exposed on the surface of their molecular structures and can recognize and bind to certain intermediates of protein substrate folding. However, the structural and functional properties allowing characterization of a protein as a chaperone are unclear.

Thus, study of chaperone-like properties of heat-stable hydrophobic proteins is of particular interest. One such protein, macrophage migration inhibitory factor (MIF), was discovered ~40 years ago and is known as cytokine. Based on its capacity to inhibit macrophage migration *in vitro*, MIF was identified as a factor produced by activated T-lymphocytes [16, 17]. Later, it was shown that low-molecular-weight highly conserved MIF protein (12.5 kD) is widespread in various biological objects (from bacteria to mammals), its maximal quantity being found in macrophages and various brain areas. Then MIF was "rediscovered" as an anterior pituitary hormone [18] and a mediator of system stress response [19, 20]. MIF was found to participate at all levels of functioning of the stress-induced hypothalamus–pituitary–adrenal system [19, 21, 22].

Based on structural and functional characteristics of MIF, it was suggested to participate in biochemical mechanisms of formation of protective reactions in an organism under heat shock resulting in denaturation and aggregation of labile proteins. The goal of this work was to study the effect of MIF isolated from bovine brain on aggregation of model protein substrates—glycogen phosphorylase *b* (Ph *b*) from rabbit skeletal muscle and yeast alcohol dehydrogenase (ADH)—in an *in vitro* test system based on kinetic studies of heat aggregation of these proteins.

MATERIALS AND METHODS

Reagents. In this study we used Tris, Hepes, α-D-glucose-1-phosphate, BSA, molecular mass markers, and *p*-hydroxyphenylpyruvate (HPP) from Sigma (USA); ADH and 5′-AMP from ICN Biomedical Inc. (USA); acrylamide, N,N′-methylenebisacrylamide, and ammonium persulfate from Serva (Germany); HW-55 Toyopearl from TOYO SODA (Japan). Other reagents were of chemically pure or analytically pure grade. Water was deionized using a Milli Q System from Millipore (USA).

MIF isolation from bovine brain. A method developed by us earlier was applied with minor modifications [23, 24]. Brain obtained from a slaughter-house was frozen and stored for a long time at −70°C. Before isolation, tissue was thawed and homogenized using a Waring blender-type homogenizer in 100 mM Tris-HCl buffer, pH 7.8 (3 : 1 v/v) containing 100 mM KCl, 5 mM β-mercaptoethanol (β-ME), and 1 mM phenylmethylsulfonyl fluoride. The homogenate was centrifuged at 10,000*g* for 30 min. Then the supernatant was centrifuged at 60,000*g* for 1 h using a Beckman L7 centrifuge (USA). The secondary supernatant was heated on a water bath for 20 min at 58°C with subsequent centrifugation at 60,000*g* for 1 h. The supernatant was salted out with ammonium sulfate to final 80% saturation. The suspension was stored for a long time at 4°C. After centrifugation of an aliquot of the protein suspension in ammonium sulfate at 12,000*g* for 20 min, the pellet was dissolved in 20 mM Tris-HCl, pH 7.0, and applied onto a Toyopearl HW-55 column (1.6 × 70 cm) equilibrated with the same buffer. The elution rate was 0.6 ml/min.

The MIF-containing fractions were identified by their keto-enol tautomerase activity, which was assayed via the rate of enol isomer formation from HPP keto isomer used as the substrate [25, 26]. Increased optical absorption at 330 nm was measured using a Beckman DU 650 spectrophotometer (USA).

Isolation of glycogen phosphorylase *b* from rabbit skeletal muscle. Ph *b* was isolated according to [27] using dithiothreitol instead of cysteine and recrystallizing the enzyme thrice. The preparation was stored at −20°C in β-glycerophosphate-NaOH buffer, pH 6.8, containing 50% glycerol and 30 mM β-ME. Then the enzyme solution was dialyzed at 4°C against 80 mM Hepes, pH 6.8, containing 0.2 mM EDTA. The yield of protein was 0.5 mg per g of raw tissue. Ph *b* concentration was determined spectrophotometrically using absorption coefficient 13.2 for 1% protein solution at 280 nm [28].

Kinetics of heat aggregation of protein substrates. To analyze heat aggregation of ADH and Ph *b*, we used turbidimetry in 20 mM Tris-HCl, pH 7.0, and 0.08 M Hepes, pH 6.8, containing 0.2 mM EDTA, respectively. Since formation of protein aggregates is followed by increased turbidity of protein solutions, kinetics of aggregation was monitored via increased apparent optical absorption at 360 nm in 1 cm-length thermostatted cuvettes using a Beckman DU-650 spectrophotometer. Buffer was preincubated at a chosen temperature (40-48°C) in the absence or in the presence of various MIF concentrations, and the aggregation process was induced by addition of protein substrate. The value of the apparent optical absorption was automatically recorded every 2 min for 30-200 min.

For calculation of molar stoichiometric ratios (MIF/protein substrate), we used molecular mass values 40, 97, and 12 kD for ADH, Ph *b*, and MIF, respectively.

Electrophoresis in polyacrylamide gel. After completion of the aggregation process, samples were centrifuged

at 10,000*g* for 45 min. The supernatant and the pellet dissolved in 20 mM Tris-HCl, pH 7.0, containing 4 M urea, 2% β-ME, and 10% SDS were analyzed by electrophoresis according to Laemmli [29] in 12% polyacrylamide gel in the presence of SDS. In several cases, the pellet was dissolved in 20 mM Tris-HCl, pH 7.0. Electrophoresis under non-denaturing conditions was performed in 10% polyacrylamide gel in buffer systems for separation of acidic and neutral proteins: for the concentrating gel, 0.06 M Tris-HCl, pH 6.7; for the separating gel, 0.37 M Tris-HCl, pH 8.9; electrode buffer, 0.05 M Tris, 0.38 M glycine, pH 8.3; voltage 300 V, currency 30 mA. The following molecular mass standards were used (kD): aldolase, 160; glycogen phosphorylase *b*, 97; BSA, 67; ovalbumin, 90 and 45; carbonic anhydrase, 29; trypsin inhibitor, 20; α-lactalbumin, 14. Protein was silver stained according to the standard procedure.

Protein concentration was determined according to Bradford [30].

RESULTS AND DISCUSSION

Isolation of MIF from bovine brain. To isolate homogeneous MIF, earlier we developed a relatively simple method including homogenization of brain tissue, centrifugation, incubation of the supernatant for 20 min at 58°C, salting-out of the supernatant with ammonium sulfate, and subsequent fractionating on a column with Toyopearl HW-55 [24]. MIF-containing fractions exhibited keto-enol tautomerase activity typical of this protein using HPP as a substrate [25, 26, 31]. As shown by SDS-PAGE, the isolated protein migrates as a single band with

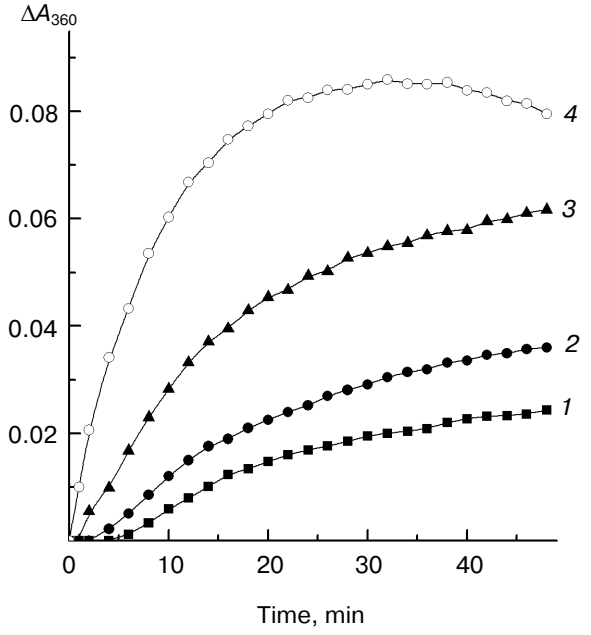

Fig. 1. Kinetic curves of ADH (70 μg/ml) aggregation in the absence (*1*) or in the presence of MIF at concentrations 3.3 (*2*), 4.7 (*3*), and 10.8 (*4*) μg/ml, registered via increase in optical absorption at 360 nm in 20 mM Tris-HCl buffer, pH 7.0, at 44°C.

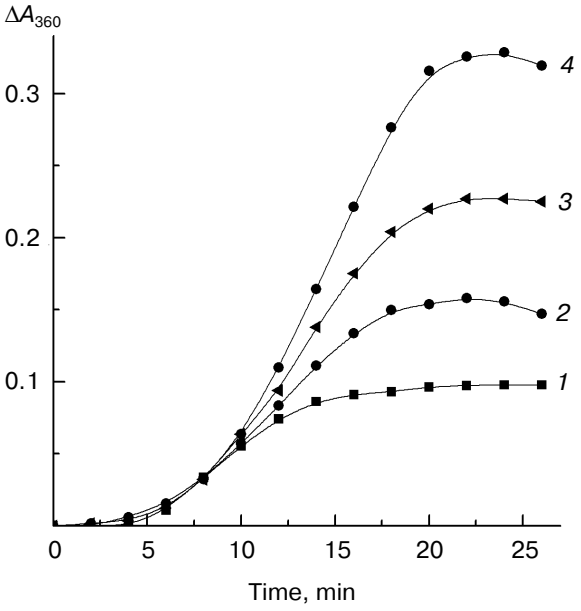

Fig. 2. Kinetic curves of Ph *b* (0.27 mg/ml) aggregation in the absence (*1*) or in the presence of MIF at concentrations 10.8 (*2*), 28.8 (*3*), and 46.8 (*4*) μg/ml in 80 mM Hepes buffer, pH 6.8, containing 0.2 mM EDTA, at 48°C.

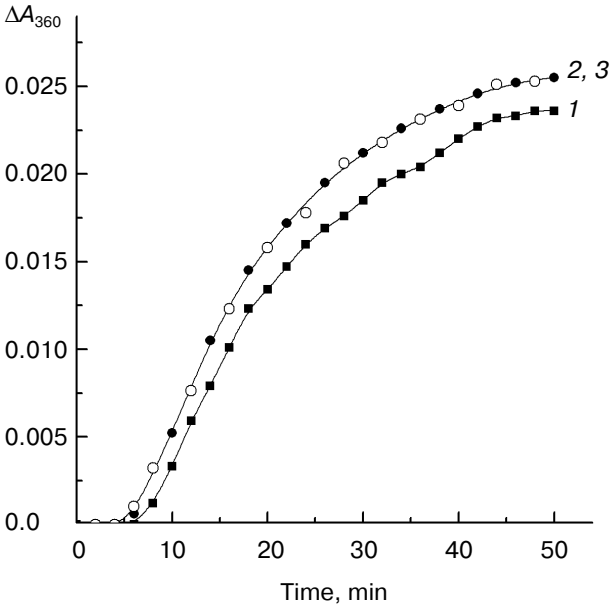

Fig. 3. Kinetic curves of ADH (70 μg/ml) aggregation in the absence (*1*) or in the presence of BSA at concentrations 30 (*2*) and 60 (*3*) μg/ml in 20 mM Tris-HCl, pH 7.0, at 44°C.

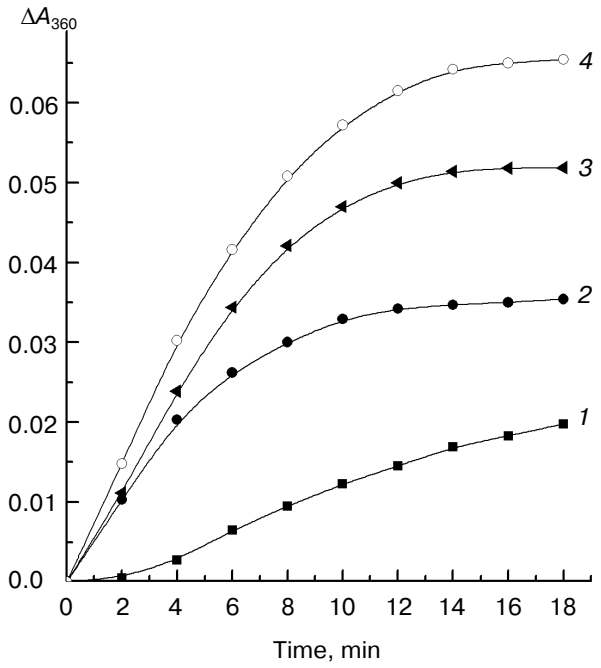

Fig. 4. Kinetic curves of ADH (70 μg/ml) aggregation in the absence (*1*) or in the presence of MIF at concentrations 3.3 (*2*), 4.7 (*3*), and 10.8 (*4*) μg/ml in 20 mM Tris-HCl, pH 7.0, containing 20 mM β-ME, at 44°C.

corresponding molecular mass 12 kD. Using immunoblotting with anti-MIF antibodies, reverse-phase HPLC, mass spectrometry, N-terminal microsequencing, and search for analogous amino acid sequence in Data Bank, it was shown that the isolated protein was identical to MIF [23, 24].

The simple and fast method for MIF isolation suggested by us is characterized by high reproducibility and relatively high yield—0.1 mg of homogeneous MIF per g of raw brain tissue.

Thermal aggregation of ADH and Ph *b* in the presence of MIF. Aggregation of ADH at 44°C was studied in the absence and in the presence of MIF at various concentrations (Fig. 1). Significant acceleration of ADH aggregation and increased limiting value of apparent optical absorption at 360 nm and $t \to \infty$ (A_{lim}) were shown to occur at sub-stoichiometric molar ratios MIF/ADH, 0.15 : 1 (curve *2*) and 0.2 : 1 (curve *3*), and at 0.5 : 1 ratio more than four-fold increase in A_{lim} value was observed (curve *4*). The lag period of the aggregation process decreased with increase in MIF concentration; in the absence of MIF this period was about 4.5 min (curve *1*).

Analogous results were obtained while studying Ph *b* aggregation (Fig. 2). At sub-stoichiometric MIF/Ph *b* ratios 0.3 : 1 (curve *2*) and 0.8 : 1 (curve *3*), a significant activating effect was observed, and on increase in MIF concentration up to MIF/Ph *b* ratio 1.5 : 1 (curve *4*) the A_{lim} value increased 3.5-fold.

These results were highly reproducible over a large number of experiments (for ADH, $n = 9$; for Ph *b*, $n = 7$). It should be noted that in the absence of protein substrates MIF did not aggregate even at concentrations one order of magnitude higher than those used in the above mentioned experiments and also on incubation for 240 min at 60°C.

Under analogous conditions, BSA at concentration 30 μg/ml had almost no effect on ADH aggregation (Fig. 3, curve *2*) and at twice increased BSA concentration (to 60 μg/ml) the result was the same (curve *3*). This indicates that the MIF effect on the aggregation process is specific. Besides this, kinetics of ADH aggregation in the presence of 20 mM β-ME (Fig. 4) and the effect of MIF on this process did not differ from the kinetics observed in the absence of β-ME (Fig. 1), all other factors being the same. The data suggest that the effect of MIF on the substrate aggregation is not mediated by formation of the S–S bonds.

For the initial analysis of aggregates after completion of the aggregation process, the samples were centrifuged and the pellets were solubilized in 20 mM Tris-HCl, pH 7.0, containing 4 M urea and analyzed by electrophoresis under denaturing conditions (Fig. 5). As a result of Ph *b* incubation in the presence of MIF at 48°C (also Fig. 2, curve *4*), Ph *b* (monomer, apparent molecular mass 97 kD) content in the pellet significantly increased (Fig. 5, lane *3*) as compared with the MIF-free sample (lane *2*). The data on ADH incubation in the

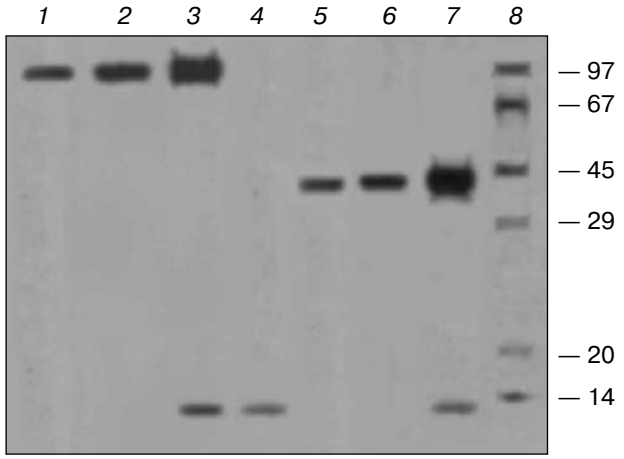

Fig. 5. Electrophoresis in 12% polyacrylamide gel in the presence of SDS of solubilized Ph *b* and ADH aggregates after completion of aggregation. Lanes: *1*, *4*, *5*) initial Ph *b*, MIF, and ADH preparations, respectively; *2*, *3*) pellets obtained by centrifugation at 10,000*g* of Ph *b* (0.27 mg/ml) suspensions after incubation in 80 mM Hepes, pH 6.8, containing 0.2 mM EDTA, for 25 min at 48°C in the absence (*2*) or in the presence of 46.8 μg/ml MIF (*3*); *6*, *7*) pellets of ADH (70 μg/ml) suspensions after incubation in 20 mM Tris-HCl, pH 7.0, for 40 min at 44°C in the absence (*6*) or in the presence of 10.8 μg/ml MIF (*7*); *8*) molecular mass markers (in kD).

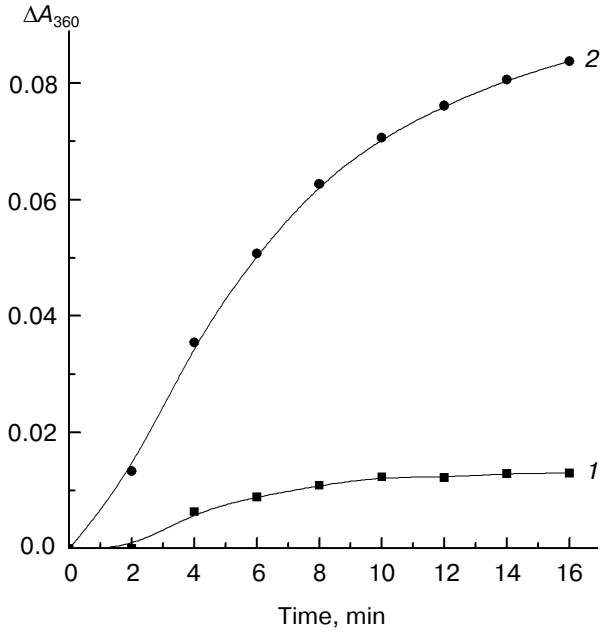

Fig. 6. Kinetic curves of ADH (0.13 mg/ml) aggregation in the absence (*1*) or in the presence of MIF (24 μg/ml) (*2*) in 20 mM Tris-HCl, pH 7.0, at 41.5°C.

presence of MIF at 44°C are analogous (also Fig. 1, curve *4*). ADH content (monomer, 40 kD) in the pellet of the MIF-containing sample (lane *7*) was much higher than in the control (lane *6*). Detection of heteroaggregates containing the substrate protein as well as MIF (monomer, 12 kD) (lanes *3* and *7*) is of great importance.

The results of electrophoresis in polyacrylamide gel under the non-denaturing conditions of supernatants (10,000*g*, 45 min) of the samples after completion of the aggregation process of Ph *b* and ADH correlate with the results of electrophoresis in the presence of SDS provided on Fig. 5. In supernatants of the samples incubated in the presence of MIF under heat stress conditions, Ph *b* or ADH content was markedly lower as compared with analogous MIF-free samples in which aggregation of the substrate proteins was less intensive (data not presented).

The data show that the presence of MIF significantly accelerated aggregation of the model protein substrates and increased the limiting value of apparent optical absorption at 360 nm and $t \to \infty$ (A_{lim}). This indicates that MIF exhibited anti-chaperone activity at temperatures exceeding the physiological level for the studied proteins.

To elucidate the effect of MIF on protein aggregation at near physiological temperature, we studied kinetics of protein aggregation at 41.5°C in the absence or in the presence of MIF. Distinct anti-chaperone MIF activity at sub-stoichiometric molar ratio MIF/ADH = 0.6 : 1 was demonstrated using ADH aggregation as an example (Fig. 6). In this experiment characterized by more moderate denaturing conditions, aggregates in pellets appeared to be easily soluble in urea-free Tris-HCl buffer.

The results of the native electrophoresis in polyacrylamide gel of ADH samples incubated in the absence or in the presence of MIF for 20 min at 41.5°C (Fig. 7) demonstrated that the supernatant contained ADH dimer and tetramer with apparent molecular masses 80 and 160 kD, respectively, the content of these oligomers being less in the presence of MIF (lane *3*) than in its absence (lane *1*). However, in the absence of MIF, in the soluble pellet we observed a faint spot corresponding to ADH tetramer and poorly gel-penetrating high-molecular-weight oligomers (lane *2*). In the presence of MIF (lane *4*), in the pellet there were observed rather bright bands, which possibly corresponded to ADH dimer and tetramer, high-molecular-weight oligomers, and also a MIF band (apparent molecular mass 12 kD). This indicates formation of heterooligomers containing ADH as well as MIF. In this case, high-molecular-weight oligomers were located in the area of lower molecular masses (lane *4*) compared with the MIF-containing sample (lane *2*).

The results of the last experiment indicate that under heat stress (temperature close to physiological level) the aggregates appear to be easily soluble, in spite of the anti-

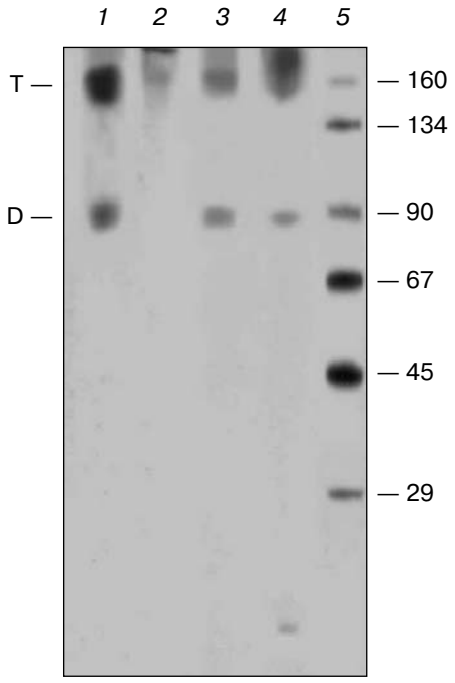

Fig. 7. Non-denaturing electrophoresis of samples after completion of the ADH aggregation process. Lanes: *1*, *3*) supernatants obtained on centrifugation at 10,000*g* of ADH suspensions after incubation in 20 mM Tris-HCl, pH 7.0, for 20 min at 41.5°C in the absence (*1*) or in the presence of 24 μg/ml MIF (*3*); *2*, *4*) pellets of the same samples in the absence (*2*) and in the presence of 24 μg/ml MIF (*4*); *5*) molecular mass markers (in kD). Positions of ADH dimer (D) and tetramer (T) are shown on the left.

chaperone MIF effect, which manifests itself in facilitated aggregation of the labile protein substrates. This can be interpreted as reversibility of the aggregation process after removal of denaturing conditions and, thus, increased probability of protein renaturation into the native state. In this sense, at the beginning of the aggregation process MIF has a fast anti-chaperone effect, reversibly binding to a partly denatured protein, stabilizing its structure and, perhaps, concentrating the substrate in the aggregated state. However, finally on removal of heat stress and restoration of the system to the normal state, MIF can play a chaperone-like role.

Analysis of kinetic curves demonstrates that the aggregation process of labile protein substrates in the presence of MIF is to a high degree cooperative. This clearly manifests itself on ADH aggregation at near physiological temperature (Fig. 6). During a comparatively short time period (about 15 min), the sigmoid aggregation shows ~10-fold increase in A_{lim} value under the influence of MIF in the sub-stoichiometric range of concentrations.

Being a thermostable hydrophobic protein, MIF is capable of binding to lipophilic proteins and peptides [32-34]. This property is also typical of other chaperone-like proteins. It is noteworthy that some known chaperones, namely protein disulfide isomerase and trigger-factor (peptidyl-prolyl-*cis-trans*-isomerase), which catalyze folding, and also HSP47, under certain conditions exhibit anti-chaperone activity [10, 35-39]. Further studies are necessary to answer the question whether MIF can function as chaperone or anti-chaperone in a multicomponent chaperone network together with other chaperones (HSP70, HSP90, or GroEL).

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