Effect of acrylamide on aldolase structure.
II. Characterization of aldolase unfolding intermediates

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Abstract

Molecules of muscle aldolase A exposed to acrylamide change their conformation via I₁, T, I₂, D intermediates [1] and undergo a slow irreversible chemical modification of thiol groups. There is no direct correlation between activity loss and thiol groups modification. In the native enzyme two classes of Trp residues of 1.8 ns and 4.9 ns fluorescence lifetime have been found. Acrylamide (0.2–0.5 M) increases lifetime of longer-lived component, yet the transfer of aldolase molecules even from higher (1.0 M) perturbant concentration to a buffer, allows regain original Trp fluorescence lifetime. I₁, detected at about 0.2 M acrylamide, represents low populated tetramers of preserved enzyme activity. T, of maximum population at about 0.7–1.0 M acrylamide, consists of meta-stable tetramers of partial enzymatic activity. These molecules are able to exchange their subunits with aldolase C in opposition to the native molecules. At transition point for I₂ appearance (1.8 M acrylamide), aldolase becomes highly unstable: part of molecules dissociate into subunits which in the absence of perturbant are able to reassociate into active tetramers, the remaining part undergoes irreversible denaturation and aggregation. Some expansion of aldolase tetramers takes place prior to dissociation. D, observed above 3.0 M acrylamide, consists of irreversibly denatured enzyme molecules. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Dynamic light scattering; Conformational change; Fluorescence lifetime

1. Introduction

Acrylamide is a neurotoxic compound altering the activity and stability of some enzymes [2–4]. It was demonstrated that model compounds of low molecular mass, containing amino and thiol groups, may react concurrently with α,β-unsaturated compounds. At comparable steric environments amino groups are about 280 times less reactive than sulfur anions [5]. Acrylamide reacts more slowly than other compounds, such as acrylonitrile or methyl acrylate [5,6].

It was demonstrated that in some proteins alkylation of cysteine residues by acrylamide yields a stable thioether derivative [7]. Acrylamide may react with highly reactive active-site Cys-149 residue of glycer-aldehyde-3-phosphate dehydrogenase [8] but it was found that acrylamide does not react with four...
exposed thiol groups of ovalbumin [9]. Bastyns and Engelborghs found partially irreversible inactivation of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) by acrylamide [10]. According to these authors, 65% of this inactivation was not caused by active-site thiol group modification.

Rabbit muscle aldolase is a tetrameric protein with 4 exposed thiol groups (Cys-72, Cys-239, Cys-289, Cys-338) per subunit [11,12] (the numbering of amino acid residues is according to [13]). These residues are localized outside the active site [14]. One group, Cys-239, is about 100 times more reactive than remaining exposed groups [11,15,16].

The enzyme functions via a Schiff base mechanism in which Lys-229 is involved in covalent binding of substrates [11].

In the preceding paper the analysis of changes of the following parameters: catalytic activity, ANS fluorescence during its displacement from the aldolase active-site, UV difference signal at 292 nm and CD spectra have shown that native (N) enzyme molecules abruptly change their structure at defined acrylamide concentration ranges. These conformational transitions are time- and acrylamide concentration-dependent. It was found that native aldolase molecules become transferred into partially active, highly populated intermediate state, T, about 1 M acrylamide, and then into inactive denatured, D, state, above 3.0 M acrylamide. It has been postulated that transitions from N to T state or from T to D state include an intermediate step I1 or I2, respectively.

In this paper, an attempt is made for closer examination of these intermediates. Particular interest was taken in the T state which exhibited partial enzymatic activity and could recover full activity after acrylamide removal. Steady-state fluorescence spectra, fluorescence lifetime measurements, light-scattering analysis and hybridization experiments were used to follow the enzyme conformational changes in the presence of acrylamide.

### 2. Materials and methods

#### 2.1. Chemicals

Potassium salt of DTNB was obtained as previously described [17]. CellULO ace atelectrophoresis strips were from Gelman Instrument Co. Remaining chemicals and the activity test were used as described in [1]. All samples were prepared in 100 mM Tris–Cl, 1 mM EDTA buffer, pH 7.5 (25°C) with or without 100 mM NaCl except where stated otherwise. Porcine brain aldolase C with a specific activity of 6–8 units/mg was obtained according to [18]. Aldolase modified with acrylamide was obtained as follows. The protein sample (ca. 0.35 mg ml⁻¹ in 100 mM Tris, 5 mM EDTA (pH 7.5), 25°C) was incubated with acrylamide (0.1–1.0 M) for 1 h at 25°C, then reaction mixture was loaded on PD-10 (Pharmacia) column and protein fractions were collected. Protein concentration of aldolase–acrylamide derivative was determined from aldolase molar absorbance coefficient at 280 nm corrected for acrylamide absorption at 280 nm.

The following absorption coefficients were used for the spectrophotometric determination of concentration: muscle aldolase, A 0.91 mg⁻¹ cm⁻¹ ml⁻¹ [19]; brain aldolase C, 0.88 mg⁻¹ cm⁻¹ ml⁻¹ [20]; TNB⁻: 13600 M⁻¹ cm⁻¹ at 412 nm [21]. The concentration of DTNB was determined from absorbance at 412 nm after reduction of DTNB with an excess of 2-mercaptoethanol.

#### 2.2. Determination of SH groups in aldolase samples

Total number of –SH groups present in native aldolase or modified with acrylamide was determined in 2% SDS (in 100 mM Tris buffer, 5 mM EDTA, pH 7.5) with 0.5 mM DNTB in the same buffer. After 15 min of the reaction with DNTB the absorbance at 412 nm was read against an appropriate blank containing 2% SDS and 0.5 mM DNTB. The kinetics of exposed –SH groups in aldolase samples was analyzed with DNTB (2 mM) and was followed on a HI-TECH SF-51 stopped-flow spectrophotometer. For each experimental curve 1024 points were collected and pseudo-first-order rate constants for fast-reacting Cys-239 and slow-reacting Cys-72, Cys-289, Cys-338 were obtained as described previously [22].

#### 2.3. Dynamic light scattering measurements

Outlines: by means of dynamic light scattering it is possible to find the actual (apparent) translational
diffusion coefficient \( D_T \) of a macromolecule in solution. Measured intensity correlation function \( g^{(2)}(t) \) for monodisperse solution can be expressed as [23]:

\[
g^{(2)}(t) = 1 + A \exp(-2q^2D_Tt)
\]

where \( A \) is a dimensionless factor (of no importance here), \( q \) is the value of the scattering vector \( q = 4\pi n \sin(\Theta/2)/\lambda \), \( n \) being the refractive index of the solvent, \( \Theta \) the scattering angle and \( \lambda \) the wavelength of laser light. If more species of molecules contribute to the light scattering the distribution of relaxation rates \( W(I) \) \((I = q^2D_T)\) or \( L(\tau) \) \((\tau = 1/I)\) is often calculated in order to resolve the species number and contribution to the scattered light. Taking into account the Stokes–Einstein relation:

\[
D_T = kT/6\pi \eta R
\]

where \( (k, \) the Boltzmann constant; \( T, \) temperature; \( \eta, \) solvent viscosity; \( R, \) hydrodynamic radius) it is possible to convert the relaxation rates distribution \( W(I) \) into apparent hydrodynamic radius distribution \( V(R_{app}) \). Combining \( V(R_{app}) \) with measured refractive index increment and the assumption that all the species are of roughly spherical shape, one can estimate their real concentrations.

Light-scattering apparatus consisted of Nd-Yag laser DPSS 532 (Coherent, USA) or Ar\(^+\) laser ILA 120 (Carl Zeiss Jena, Germany), optical setup with a thermostatic sample holder, Thorn EMI 9130 B03100 photomultiplier, ALV/PM-PD preamplifier/discriminator (ALV, Germany), and ALV-5000 digital structurator/correlator (ALV, Germany). Measurements were performed at \( \lambda = 488 \) nm or 532 nm (dependent on the laser used) and \( \Theta = 90^\circ \). All the solutions were filtered with syringe filters (0.2 \( \mu \)m) (Millipore Co., USA) and centrifuged in quartz cuvette not less than 10 min at 15 000 \( \times g \) before measurements. The scattering intensity signal was equilibrated for at least 10 min. The temperature was controlled with an accuracy of \( \pm 0.1^\circ C \) and measured with a thermocouple. Relative viscosities were measured using capillary viscosimeter of Ubbelhode type. Refractive indexes were measured with Abbe refractometer. Both static and dynamic light scattering information was used in data interpretation. Measured correlation functions were analyzed by means of the computer program CONTIN [24], which calculates the distribution of relaxation rates. These results were then combined with the information of total scattered light intensities.

2.4. Hybridization experiments

Equal protein samples (0.2 mg ml\(^{-1}\)) of rabbit muscle aldolase A and porcine brain aldolase C were incubated with or without acrylamide (0.7, 1.2 or 1.8 M) at 25\(^\circ\)C in the buffer containing NaCl. After 20 min, samples were diluted (1:10) in the buffer containing 0.5 mg/ml BSA. Then they were passed through a PD-10 column equilibrated with the buffer containing 5 mM 2-mercaptoethanol. The samples were incubated for 1 h at 0\(^\circ\)C and then they were concentrated on Microcon-10 membranes (Amicon, USA) (ca. 1:250) at 4\(^\circ\)C. Finally the samples were centrifuged at 25 000 \( \times g \) at 4\(^\circ\)C for 60 min and 4 \( \mu \)l aliquots were applied on cellulose acetate strips. Electrophoresis and aldolase activity staining was performed according to [25]. The control sample (showing hybrids) was obtained by reversible dissociation of the mixture of aldolase A and aldolase C with citric acid (pH 2.3) according to [26].

2.5. Fluorescence lifetime measurements

The fluorescence lifetime of the tryptophan residues of aldolase was measured with SLM-Aminco 48000S spectrofluorometer. POPOP in methanol, Trp or indole in water were used as reference samples (\( \tau = 1.35 \) ns, 2.5 ns, 4.1 ns, respectively). The emission (\( \lambda_{ex} = 295 \) nm) was detected through WG-320 (Schott) filter (cut-off 320 nm) placed between the sample and the emission photomultiplier tube (PMT). Multifrequency analysis and distributed lifetime calculation were applied to the analysis of heterogeneous fluorescence lifetimes from the phase shift and amplitude modulation data in the range of frequencies from 10–250 MHz with the SLM software. The measurements were performed in the 10 mM Tris–Cl, 1 mM EDTA, 100 mM NaCl buffer at 25\(^\circ\)C for: (i) native aldolase (5–8 \( \mu \)M), (ii) aldolase modified with acrylamide obtained after 1 h incubation of aldolase (ca. 10 \( \mu \)M) with 1 M acrylamide and subsequent removal of unreacted acrylamide on PD-10 (Pharmacia) column (final concentration of modified aldolase was 5–8 \( \mu \)M), (iii) aldolase (5–8 \( \mu \)M) in the presence of acrylamide (0.2 or 0.5 M).
The start of data acquisition was performed 10 min after the addition of acrylamide (iii) or 30 min after the removal of acrylamide (ii), and the measurements were completed after 30–60 min. At least three separate samples were measured to determine fluorescence lifetimes and fractional contributions.

3. Results

3.1. Acrylamide effect on the aldolase SH groups

Since –SH groups are the potential candidates for the reaction with acrylamide, the effect of acrylamide on aldolase thiols was investigated. As shown in Table 1 incubation of aldolase with 0.1 M acrylamide for 90 min results in ca. 14% decrease of aldolase activity. No detectable decrease of aldolase thiol groups, within experimental error range, was observed at these conditions. Higher acrylamide concentration causes a further decrease of aldolase activity and partial reduction of the number of exposed thiols, e.g., 75% of highly reactive cysteine residues and ca. 15% of slowly reactive thiols were lost in the presence of 0.5 M acrylamide. After prolonged incubation of aldolase with 1 M acrylamide approximately one fast-reacting group and two of three exposed slowly reacting thiols were modified. These data indicate that four buried groups per subunit were not affected by acrylamide. After the removal of an excess of acrylamide, the modified enzyme recovered 90% of its initial activity. All these results indicate lack of a direct correlation between thiol groups modification and enzyme activity.

Table 1

<table>
<thead>
<tr>
<th>Acrylamide concentration (M)</th>
<th>Fast-reacting Cys</th>
<th>Slow-reacting Cys</th>
<th>Total Cys</th>
<th>Activity (%)</th>
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<tr>
<td></td>
<td>number per subunit</td>
<td>rate const. (s⁻¹)</td>
<td>number per subunit</td>
<td>rate const. (s⁻¹)</td>
</tr>
<tr>
<td>0.0</td>
<td>0.98</td>
<td>0.1</td>
<td>3.4</td>
<td>0.001</td>
</tr>
<tr>
<td>0.1</td>
<td>0.95</td>
<td>0.1</td>
<td>3.4</td>
<td>0.001</td>
</tr>
<tr>
<td>0.5</td>
<td>0.25 ± 0.2</td>
<td>0.085</td>
<td>2.9</td>
<td>0.001</td>
</tr>
<tr>
<td>1.0</td>
<td>0.0</td>
<td>0.0</td>
<td>1.07</td>
<td>0.001</td>
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</tbody>
</table>

2 μM aldolase solution in 100 mM Tris, 1 mM EDTA buffer (pH 7.5) at 25°C was incubated for 90 min with indicated acrylamide concentrations and enzyme activity was determined. Number of –SH groups was determined with Ellman reagent 90 min after removal of acrylamide on PD-10 column as described in Section 2.

3.2. Fluorescence spectra and fluorescence lifetimes measurements

The fluorescence emission spectrum of native aldolase excited at 280 nm has its maximum at 328 nm [27]. In aldolase treated with 0–0.7 M acrylamide, no red shift in the position of the fluorescence maximum, typical of the process of denaturation, was observed (not shown). However, prolonged incubation of aldolase in 4 M acrylamide shifts the fluorescence maximum from 328 nm to 340.5 nm, as shown in Fig. 1. The intensity of fluorescence decreases with time. Incubation of 24 h causes an increase of the background fluorescence intensity presumably due to the slow precipitation of the enzyme (see Sections 3.3 and 4).
We found that under all conditions the fluorescence decay of the tryptophan residues in aldolase had to be fitted by three exponential components (Table 2). In the native enzyme three fluorescence lifetimes were observed: 1.8 ns ($f_1 = 0.66$) and 4.9 ns ($f_1 = 0.195$) originated from tryptophan residues, and the third, 2.3 ps ($f_1 = 0.145$) component caused probably by the scattered light. The presence of 0.2 M or 0.5 M acrylamide in aldolase sample resulted in the increased value of the longer component (ca. 8.5 ns) and in consequence increased values of average fluorescence lifetime (Table 2). The fluorescence lifetime of aldolase modified after removal of acrylamide consisted of two main components similar to that found for native protein.

3.3. Dynamic light scattering studies

Typical investigations within the dynamic light scattering phenomenon involve measurements of translational diffusion coefficient ($D_T$). At the same time total scattered light intensity is measured. Since aldolase molecules are charged at pH 7.5, their interactions may lead to a concentration dependence of the measured diffusion coefficient. For that reason $D_T$ was measured as a function of aldolase concentration in moderate ionic strength buffer (100 mM NaCl). $D_T$ value extrapolated to $c = 0$ was used for the estimation of aldolase molecule Stokes radius $R$ (Fig. 2). Taking measured viscosity of 0.9304 cP and the refractive index of 1.3404 we found $R = 45.7 \text{ Å}$.

Experiments in the presence of acrylamide were performed using aldolase concentration in the range from 0.5 to 1 mg ml$^{-1}$. Viscosity of the solution in the presence of acrylamide (0.0 M to 4.0 M) changes nearly linearly from 0.9304 cP to 1.614 cP, while refractive index changes linearly from 1.3404 to 1.3827 (not shown).

The effect of acrylamide (0–1.8 M) on the aldolase molecules Stokes radius is shown in Fig. 3. Average values of the data fitted to the second degree polynomial are represented with the curve starting with the radius about 45.6 Å, for native enzyme, attending minimum 43.4 Å at 0.75 M acrylamide and rising to 50 Å at 1.8 M acrylamide. Although the increase from 43.4 to 50 Å is beyond an experimental error,

<table>
<thead>
<tr>
<th>Table 2</th>
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<tr>
<td>Fluorescence lifetime parameters of aldolase tryptophan residues$^a$</td>
</tr>
<tr>
<td>Native aldolase</td>
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<tr>
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<tr>
<td>$\tau_1 = 1.8$ ns (0.66)</td>
</tr>
<tr>
<td>$\tau_2 = 4.9$ ns (0.195)</td>
</tr>
<tr>
<td>$\tau_3 = 2.3$ ps (0.145)</td>
</tr>
<tr>
<td>$\langle \tau \rangle = 2.1$ ns</td>
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$^a$All the measurements were performed with aldolase samples of final concentration 5–8 μM in 10 mM Tris, 1 mM EDTA, 100 mM NaCl (pH 7.5) at 25°C. Numbers in parentheses correspond to the fractional contribution ($f_i$) of the component with that specific lifetime ($\tau_i$). The goodness of fit between the observed and computed values of phase angle ($\Phi$) and modulation ($m$) was assessed from the value of $\chi^2 = 1/n \Sigma [(\Phi_0 - \Phi_i)/\sigma_\Phi^2 + (1/n \Sigma (m_0 - m_i)/\sigma_m^2)^2$, where $n$ is the number of degrees of freedom, $\sigma_\Phi$, $\sigma_m$ are the estimated uncertainties of phase angle and modulation, respectively. Average fluorescence lifetime: $\langle \tau \rangle = \Sigma f_i \tau_i/\Sigma f_i$. 

Fig. 2. Dependence of apparent Stokes radius on aldolase concentration (in the 100 mM Tris buffer, 1 mM EDTA, 100 mM NaCl (pH 7.5) at 25°C). Aldolase hydrodynamic radii were calculated according to Eq. 2 from diffusion coefficients measured with dynamic light scattering technique as described in Section 2. Each bar represents ± S.D. for three determinations.
the decrease from 45.6 Å to 43.4 Å is within the error range. This does not allow to conclude that shrinking of aldolase molecule precedes its expansion.

Hydrodynamic radius distributions of aldolase molecules incubated for 20 min with 0.0, 0.7, 1.8, and 3.5 M acrylamide in the presence and absence of 100 mM sodium chloride are shown in Fig. 4. Results of the CONTIN program calculations were multiplied by the total scattered intensity so that the numbers on the y-axis can be directly compared between different lines. In the analysis of these curves one should keep in mind that large molecules scatter more strongly at the same weight concentration so the distributions presented in Fig. 4 do not represent the real concentrations of different species. As a very rough estimation one can take the intensity proportional to the molecular mass, which leads to the weighing factor of $R^{-3}$. Multiplication by this factor will of course favor smaller molecules, reflecting their real contribution to the total weight concentration.

No effect of sodium chloride on the structure of native aldolase was observed (Fig. 4A). However, in the presence of 0.7 M acrylamide a population of aldolase molecules corresponding to tetramers, with $R_{\text{app}}$ about 43 Å, is distributed in a more narrow range of $R_{\text{app}}$ values in the presence of the salt than in its absence (Fig. 4B). At higher perturbant concentrations the process of the formation of high molecular mass particles is partially inhibited by NaCl because there is much less intensity of light scattered by big particles ($R > 200$ Å) (Fig. 4C,D).

In 1.8 M acrylamide, in the absence of sodium chloride, two populations of aldolase molecules were clearly visible: corresponding to the native tetrameric form of enzyme and high molecular mass particles, for peaks centered at 50 Å and 800 Å, respectively (Fig. 4C). This experiment indicates that in the presence of higher concentrations of acrylamide aldolase molecules aggregate. In control experiments performed in the absence of the protein, aggregates were not observed in the acrylamide solution (not shown). Time-dependent analysis of the changes of the hydrodynamic radius of aldolase ag-
gregates shows that in 1.2 M acrylamide aggregation in nearly completed after 170 min (Fig. 5A). It is noteworthy that under these conditions molecules of aldolase tetramers of about 48 ± 2.5 Å are still present in the solution (Fig. 5B) with no $R_{\text{app}}$ time variation.

The distribution of apparent hydrodynamic radius as a function of incubation time in 3.5 M acrylamide in buffer without NaCl is shown in Fig. 6. Starting from the left, the first peak of the lowest $R_{\text{app}}$ represents acrylamide (Fig. 6B–E), the second peak represents native aldolase (Fig. 6A) and the third peak corresponds to aldolase aggregates (Fig. 6B–E). An increase of $R_{\text{app}}$ of aggregates is again clearly visible. A decrease of an average value of apparent hydrodynamic radius of molecules initially representing aldolase tetramers by ca. 20% was observed after 2 h of incubation with 3.5 M acrylamide (Fig. 7). This result should be treated rather qualitatively because of strong increase of light scattering induced by aldolase aggregates and in consequence increase of standard deviation. Accuracy of the measurement of $R_{\text{app}}$ using scattering technique increases with time. The quality of the data presented in Fig. 7 could not be improved upon time because more and more aggregates of $R_{\text{app}}$ ca. 6800 Å were formed in the presence of small enzyme molecules possessing $R_{\text{app}}$ between 36 Å to 47 Å. Calculated with Garcia de la Torre program [28], the value of $R_{\text{app}}$ shows that in concentrated acrylamide solution some part of aldolase molecules probably dissociate into subunits and appear in a dynamic equilibrium with the aggregated form of aldolase molecules. Moreover, the intensity of scattered light of aldolase molecules in 3.5 M acrylamide is several times lower than the native enzyme in the buffer (not shown).
3.4. Hybridization studies

Interspecies hybrid formation induced by low pH was previously observed for rat aldolase A and rabbit aldolase C [29] and for aldolases from other species as well [18,30]. In order to prove, in an independent way, that rabbit aldolase A molecules may dissociate in the presence of acrylamide, hybridization experiments between aldolase A and porcine aldolase C were performed. An example of such experiment is shown in Fig. 8. Incubation of aldolase C with 1.2 M acrylamide (Fig. 8a) or incubation of the mixture of aldolase A with aldolase C without acrylamide (Fig. 8b) showed only parental bands: C₄ or both A₄ and C₄, respectively. As expected, in the control a five-membered set containing three hybrids was obtained after induced dissociation and reassociation of the mixture of the two parental aldolases (Fig. 8c).

However, in the presence of 0.7, 1.2, 1.8 M acrylamide the mixture of aldolase A and C yielded a small amount of active A₃C, A₂C₂ and AC₃ hybrids in addition to the heavy bands representing parental aldolases (Fig. 8d–f). Activity staining revealed parental aldolases first, and then, after prolong exposure, hybrids were visualized (A₃C > A₂C₂, AC₃) in the samples incubated with acrylamide, as follows: 1.8 M > 1.2 M > 0.7 M (not shown). This indicates that in the applied experimental condition, aldolase molecules were able to exchange their subunits and form active tetramers after the removal of perturbant molecules.

4. Discussion

4.1. The effect of acrylamide on aldolase thiol residues

It has been previously shown that the modification of thiol residues in aldolase A molecule affect both enzyme activity and stability. This effect depends on the chemical nature of the modifying reagent and the...
extent of modification [12,14,31], e.g., modification of highly reactive Cys-239 does not affect the enzymatic activity and stability as well [12,14] whereas for three slowly reactive Cys-72, -289, -338 a correlation between -SH group modification with DTNB and the activity loss was observed [14]. However, the replacement of all TNB residues by -CN groups results in the restoring of more than 50% of the enzyme activity [14]. The substituent (RCH\_2CH\_2S) with the R positive charged amino group mostly destabilized the aldolase molecule in comparison with neutral carbamyl or negative carboxyl group [12]. Recently, it has been shown that the modification of Cys-72 with oxidized glutathione of only a single aldolase subunit is sufficient to abolish activity in all subunits [32]. All this indicates how complex might be the effect of interaction of modified side residues in flexibility of aldolase molecule required for catalysis and enzyme stability.

We found that in the presence of 1 M acrylamide one fast-reacting cysteine and only two of the three slow-reacting thiols per aldolase subunit are lost after 90 min prolonged incubation (Table 1). This result is in the range of expectation because a comparison of previously published data on -SH groups reactivity indicates that the reaction of acrylamide with thiol anion is at least six orders of magnitude slower than the reaction of cysteine thiol with DTNB [5,15] or two to four orders of magnitude slower in comparison to the reaction of the aldolase exposed thiols with DTNB [15]. The 90 min modification of aldolase cysteine residues by acrylamide does not seem to be a direct cause of the loss of enzyme activity, because the subsequent dilution of aldolase recovers 90% of its initial activity whereas thiols remain blocked (Table 1). Moreover, most of our experiments were performed after 20 min incubation of aldolase with acrylamide. All this indicates that in low concentrations of acrylamide (up to 0.7 M) and a short 20 min exposition of aldolase to acrylamide noncovalent interactions between acrylamide and enzyme play a predominant role in conformational changes.

We are fully aware, however, that at higher acrylamide concentrations further disruption of aldolase structure occurs which may lead to the exposure of enzyme thiol groups, their modification and the enzyme structure destabilization.

### 4.2. Fluorescence lifetime measurements

Because of the variety of different elements that can alter the lifetime value of the tryptophan residue, it is difficult to described satisfactorily the emission decays. Mono- and double-exponent fits gave significantly higher $\chi^2$ values than those presented in Table 2. Because of the weak fluorescence signal we could not use polarization filters to eliminate scattered light, which probably led to the generation of a picosecond component. The analysis of the fluorescence decays using continuous lifetime distribution approach gave similar values of the fluorescence lifetimes as obtained for discrete exponential components, sometimes with lower $\chi^2$ but negative preexponential factors (not shown). The observed rather high $\chi^2$ values of fluorescence lifetime in the native enzyme may originate from the environmental heterogeneity of aldolase tryptophan residues. This heterogeneity seems to be related not only to the presence of three tryptophan residues in the enzyme subunit but also to the chosen temperature (25°C) of all the experiments described in this paper. The selected temperature was close to the temperature-induced conformational transition of the aldolase molecule at which fluorescence intensity of aldolase exhibits a break (26–27°C) [22]. Therefore, our fluorescence lifetime measurements should be treated cautiously and rather qualitatively. However, the average fluorescence lifetime of native enzyme measured in our laboratory (2.14 ns, Table 2) is in the range of values published previously: 1.55 ns and 2.3 ns by [33] and [27], respectively. If we assume that each fluorescence lifetime component originates from a different class of tryptophan residues, our results indicate the presence of two classes of tryptophan residues in the molecule of native aldolase. Presumably the shorter lifetime ascribes two tryptophan residues per subunit (fractional contribution 0.66), whereas only one Trp residue for the longer component (fractional contribution 0.195). In the presence of acrylamide increased values of $\chi^2$ were always observed, presumably due to the weak fluorescence of quenched fluorophores and strong absorption of acrylamide.

Partial modification of enzyme sulfur-bridged with acrylamide does not cause significant changes in aldolase structure since values and the distribution of
fluorescence lifetimes are similar in the native protein and the modified aldolase after removal of pertur- bant. It seems that in the presence of acrylamide, one Trp residue changes its microenvironment. This is in agreement with the results obtained from steady-state quenching experiments where fraction of fluorophore, $f_\lambda$, was changed from 0.89 to 0.59 [1]. Increased to 8.5 ns, the fluorescence lifetime of the long-lived component may be caused by the more hydrophobic microenvironment of Trp residue, which is probably not accessible to quencher.

**4.3. Light-scattering measurements**

From our light-scattering measurements the value $R$ for native aldolase molecule amounted to 45.7 Å. Using the gel filtration technique and assuming slightly lower than in reality the molecular mass of aldolase (150 kDa), $R = 45$ Å was obtained [34]. The dimensions of aldolase molecule $90 \times 90 \times 65$ Å were deduced from X-ray crystallographic studies [35]. Thus the $R$ value found from light scattering can be used as an appropriate approximation of an average radius of the aldolase molecule and for investigation of its relative changes.

In the presence of 3.5 M acrylamide a ca. 25% decrease of the aldolase apparent hydrodynamic ra- dius can be related to the dissociation of tetrameric molecules.

**4.4. Characterization of unfolding intermediates**

The subject of our studies is particularly complex because global conformational changes of aldolase molecules are associated with the exposure of new groups, leading to enzyme dissociation and/or aggre- gation in time and the acrylamide concentration de- pendent manner. Therefore unequivocal character- ization of transiently formed intermediates is rather difficult.

The intermediates can be characterized in the fol- lowing manner. (i) $I_1$ appears at about 0.2 M acryl- amide and consists of ca. 22% population of whole aldolase molecules at this acrylamide concentration [1]. It seems that it represents a short-lived interme- diate of tetrameric structure and perhaps of a slightly distorted active site. Further characterization of this state was not possible with the applied detection methods. (ii) $T$ appears at 0.4–1.2 M acrylamide. At about 1.0 M acrylamide this state represents almost 100% of the population after 20 min of aldolase exosition to acrylamide [1]. In this report it is shown that with an experimental error aldolase mole- cules do not change their Förster distance up to about 1.0 M acrylamide (Fig. 3). However, an increase of the Förster distance distribution between extrinsic fluorophore probes in aldolase molecules in the presence of 0.7 M acrylamide [36] clearly indicates that the structure of aldolase in $T$ state is strongly per- turbed. It seems that the enzyme structure fluctuates in some local minimum because the catalytic activity of aldolase, although lower than that of native mole- cules, stands at a similar level between 0.4–1.2 M acrylamide, i.e., as long as the radius of aldolase molecules does not exceed its original dimension (45.7 Å). Thus aldolase molecules of $T$ state seem to be meta-stable tetramers which spontaneously are converted into high molecular mass aggregates in a cooperative manner (Fig. 5A). This indicates that after the formation of nucleation centers, the process of aggregation occurs rather fast; however, its kinetics has not been investigated in detail. It is possible that the process of aggregation proceeds via transient appearance of the free enzyme subunits that are able to dissociate from aldolase tetramers even at 0.7 M acrylamide as shown from our hybridization experiments (Fig. 8d). Reactivation experiments per- formed on aldolase preparation incubated at differ- ent acrylamide concentrations clearly indicate that aldolase molecules of $T$ state are able to recover nearly full enzymatic activity after perturbant remov- al. (iii) $I_2$ appears between 1.2–3.0 M acrylamide. This state is highly unstable. The maximum of its population, ca. 50%, was postulated at about 2.0 M acrylamide. At 1.8 M acrylamide, i.e., at midpoint of appearance of $I_2$, aldolase molecules are strongly sus- ceptible to aggregation (Fig. 4C). This concentration of perturbant clearly induces dissociation of aldolase tetramers into monomers as judged from light-scatter- ing and hybridization experiments. Transiently formed subunits are able to refold into active inter- species hybrid tetramers in the absence of acrylamide (Fig. 8f). However, most of them are irreversibly transformed into inactive molecules, mostly aggre- gates. These conclusions are supported by light-scatter- ing data (Fig. 4C) and the reactivation experiment
which shows that aldolase incubated with 2.0 M acrylamide is able to recover only a small part of its activity. (iv) D occurs above 3.0 M acrylamide. Fluorescence spectra, CD analysis and light-scattering experiments showed that at this concentration of acrylamide aldolase molecules become randomly unfolded, susceptible to aggregation and irreversibly denatured. Interestingly, at 3.0 M acrylamide, the susceptibility of the polypeptide chains of aldolase to aggregation decreases in comparison with 1.8 M acrylamide as judged from light-scattering experiments (Fig. 4C,D). Examples of a critical concentration of denaturant, for which irreversibility of denaturation was observed, e.g., for elastase denatured with Gd-HCl the critical concentration equals 1.9 M [37]. The inhibition of aggregation of aldolase molecules, which we observed in the presence of sodium chloride, indicates that specific intramolecular hydrophobic interactions stabilize aldolase molecule integrity thus preventing unspecific association and aggregation.

A tentative scheme of aldolase conformational transitions induced by acrylamide is presented in Fig. 9. The above results indicate that the acrylamide molecule, due to its lipophilic part and its amide group, might act as a strong perturbant of protein structure, competing both for hydrophobic interactions and/or hydrogen bonding.

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References


