SITE-SPECIFIC MODIFICATION OF RABBIT MUSCLE ALDOLASE WITH FLUORESCENT PROBES*  

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The site-specific modification of rabbit muscle aldolase A by labeling of thiol residues of Cys-289 with 5-(2-((iodoacetyl)amino)ethyl)amino)naphthalene-1-sulfonic acid and Cys-239 with 5-iodoacetamidofluorescein or 4-dimethylamino-phenylazophenyl-4'-maleimide has been described. The method is based on the differences in kinetics of the chemical modification of aldolase thiols with the above reagents either in the presence or in the absence of a competitive inhibitor. The spectral properties of the doubly labeled aldolase derivatives were compared with those of the singly labeled enzyme. The doubly labeled aldolase derivatives exhibited full catalytic activity.

1. At present there is a growing interest in specific labeling of protein molecules with fluorescence energy donor and acceptor probes. Such labeled proteins are used as a tool in resonance energy transfer studies, when spatial relationships between groups are of particular interest.

A molecule of rabbit muscle aldolase consists of four identical or nearly identical subunits [1-4]. Each subunit of the enzyme contains eight SH groups. Four of them, Cys-72, Cys-239, Cys-289 and Cys-338 were classified as exposed [5, 6]. Among the four exposed thiol residues one exhibits a high reactivity (Cys-239) [5-8]. The second-order rate constant for the reaction of Cys-239 with Nbs₂ \(^1\) is 262 \(M^{-1}s^{-1}\), whereas the rate constant for the reaction of

\(^1\) Abbreviations: DABMI, 4-dimethylaminophenylazophenyl-4'-maleimide; DMF, dimethylformamide; hexitol-1,6-P₂, hexitol-1,6-bisphosphate; IAEDANS, 5-(2-((iodoacetyl)amino)ethyl) amino)naphthalene-1-sulfonic acid; IAF, 5-iodoacetamidofluorescein; DTT, dithiothreitol; Nbs₂, 5,5-dithiobis(2-nitrobenzoic acid); Nbs, 5-thio-2-nitrobenzoate; aldolase-Cys-289-AEDANS, aldolase labeled with IAEDANS at Cys-289; aldolase-Cys-239-IAF, aldolase labeled with IAF at Cys-239; aldolase-Cys-239-DABMI, aldolase labeled with DABMI at Cys-239.  

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Cys-72, Cys-289 and Cys-338 is 2.5 M$^{-1}$s$^{-1}$ [7]. Two exposed SH groups (Cys-72 and Cys-338) are protected by substrate and competitive inhibitors from chemical modification [5]. These cysteine residues are not catalytically functional and the inactivation of the enzyme caused by the sulfhydryl group modification can be attributed to steric hindrance of a catalytic site by the modifying agents [9]. Basing on the differences in sulphydryl-group reactivity with Nbs$_2$ we attempted to label specifically two sites on the aldolase molecule at Cys-239 and Cys-289 with the fluorescence energy donor (IAEDANS) and the fluorescence energy acceptor (DABMI or IAF).

The choice of these cysteines for chemical modifications was stimulated by recent observations from our laboratory which have shown that Cys-239 is located in the region sensitive to the conformational changes induced by temperature or by substrate analog binding, whereas Cys-289 is located outside of this region [10, 11]. These pairs of fluorescent labels can be used for the determination of the distance between Cys-239 and Cys-289 and may allow to understand more details of the solution-state structure of the aldolase molecule. Recently, the distance between Lys-107 at the aldolase active site region and Cys-239, was measured [12]. In this communication we describe specific labeling of aldolase with two donor-acceptor pairs, because in the distance measurements, estimated from Förster's theory, for a single donor-acceptor pair there is a high probability of obtaining erroneous results.

This communications presents the elaborated procedures for specific modification of Cys-239 with DAMBI or IAF and Cys-289 with IAEDANS.

**MATERIALS AND METHODS**

2.1. Chemicals. Aldolase with a specific activity of 14-15 units/mg was prepared as previously described [13]. The aldolase activity was assayed spectrophotometrically at 25°C in 100 mM Tris, 1 mM EDTA (pH 7.5) using a coupled enzyme assay [14]. The enzyme concentration was calculated assuming an A$_{1cm}$ value of 9.1 [15].

Hexitol-1,6-P$_2$ was prepared by the reduction of fructose-1,6-bisphosphat with sodium borohydride [16]. The concentration of hexitol-1,6-P$_2$ was determined by an analysis of phosphate content in the sample before and after hydrolysis [17].

Nbs$_2$ (Sigma) before use was converted into potassium salt [18].

IAF, DABMI and IAEDANS were purchased from Molecular Probes, Inc. (Eugen, U.S.A.).

All other chemicals used were of the best available commercial grade.

2.2. Spectrophotometric and fluorescence measurements. The absorption spectra were measured with an ACTA M VI spectrophotometer (Beckman) at 25°C.
The fluorescence measurements were made with a Perkin-Elmer MPF-44 fluorimeter equipped with corrected spectrum accessories in a 3 mm optical path microcuvette at 25°C.

2.3. Determination of exposed SH groups. A hundred microliters of 9 mM Nbs₂ was added to 1 ml of aldolase solution (2.5-3.0 × 10⁻⁶ M). The reaction was followed by monitoring the increase of absorbance at 412 nm with a reference solution of 1 ml 10 mM Tris buffer, 1 mM EDTA, pH 7.5 (stock buffer) containing the same amount of Nbs₂. After a few minutes the number of modified fast-reacting SH groups (Cys-239) was obtained by extrapolating the linear increase of absorbance back to zero time [10]. A value of 13 600 M⁻¹s⁻¹ at 412 nm for the molar absorption coefficient for Nbs was used [19].

2.4. Column chromatography. Aldolase and aldolase derivatives were separated from the low molecular weight reagents on a Sephadex G-25 coarse column (2.1 × 19 cm) equilibrated with stock buffer [10 mM Tris buffer, 1 mM EDTA, pH 7.5] (25°C). After the application of a 2 ml aldolase sample on to the top of the column, 1 ml fractions were eluted with the same buffer and assayed for activity and absorption at 280 nm.

2.5. Chemical modifications. The following scheme was used for the preparation of aldolase labeled on Cys-289 with IAEDANS and labeled on Cys-239 with IAF or DABMI: 1, protection of Cys-72 and Cys-338 against chemical modification by saturation of the aldolase active site with the substrate analog, hexitol-1,6-P₂ according to [5]; 2, modification of Cys-239 with Nbs₂; 3, labeling of Cys-289 with IAEDANS; 4, deprotection of Cys-239 by reaction with DTT; 5, labeling of aldolase-AEDANS with IAF or DABMI at Cys-239 in the presence of a competitive inhibitor.

This seemingly complicated route of modification takes into consideration the well known chemistry and kinetics of the reaction of aldolase thiols with Nbs₂ in the presence and absence of substrate analogs [5-8]. We felt that it is much simpler and safer for the final success of specific modification of aldolase to start with the aldolase-Cys-239-Nbs derivative containing only one thiol (Cys-289) available for further modification exposed, in the presence of a competitive inhibitor, than to study the kinetics of the aldolase modification with different fluorescent labels and to adopt possible results for finding the best conditions for direct and specific modifications.

2.5.1. Preparation of aldolase-Cys-239-Nbs derivative. In the spectrophotometer cuvette 20 µl 50 mM hexitol-1,6-P₂ was mixed with 1 900 µl of aldolase (3-4 × 10⁻⁵ M) in the stock buffer [containing 10 mM Tris, 1 mM EDTA (pH 7.5)]. 1 900 µl of the above mentioned buffer containing 20 µl of 50 mM hexitol-1,6-P₂ was added to a reference cuvette. Then 100 µl of 9 mM Nbs₂ was added to each cuvette and the absorbance at 412 nm was monitored. After a few minutes, when the absorbance reached a value corresponding to the modification of about 1.0 SH group per aldolase subunit, the reaction was stopped by application of the mixture to a Sephadex G-25
column (see section 2.4). The exact number of Nbs residues bound per protein molecule in the Nbs derivative of aldolase was determined by measurements of the absorbance at 280 nm and 330 nm according to the method of Yun & Suelter [20], taking as absorption coefficients values of 3 560 M$^{-1}$cm$^{-1}$ and 8 750 M$^{-1}$cm$^{-1}$ at 280 nm and 330 nm, respectively.

As a control the deprotection of modified aldolase with DTT (20 µl 1 M per 1 ml of the protein sample) was followed at 412 nm. When absorption reached a constant value (after 15-20 min) it was assumed that all the Nbs groups are free in the solution and their concentration was calculated according to the Ellman method [19]. The protein concentration in the aldolase-Nbs sample was estimated as in the Yun & Suelter method [20], i.e. the absorbance of modified sample was measured at 280 and 330 nm, and the aldolase concentration, $c_{ald}$, was corrected for the Nbs absorption according to equation (1):

$$c_{ald} = \left[ A_{280} - \left( \frac{A_{330}}{\varepsilon_{Nbs}^{280}} \times \varepsilon_{Nbs}^{330} \right) \right] / \varepsilon_{280}^{ald} \times M_w^{ald}$$  

(1)

where $A_{280}$ and $A_{330}$ are the absorption values at 280 and 330 nm, $\varepsilon_{Nbs}^{280}$, $\varepsilon_{Nbs}^{330}$ and $\varepsilon_{280}^{ald}$ are the molar absorption coefficients for the Nbs derivatives at 280 and 330 nm, and the aldolase molar absorption coefficient at 280 nm, respectively; $M_w^{ald}$ is the aldolase molecular weight.

2.5.2. Reaction of aldolase with IAEDANS at Cys-289. A hundred microliters of 50 mM hexitol-1,6-P$_2$ was added to 5 ml of aldolase-Cys-239-Nbs derivative (2-5 × 10$^{-6}$ M) in the stock buffer. This mixture was allowed to react in the dark with 5 ml of 2 mM IAEDANS in stock buffer [10 mM Tris, 1 mM EDTA (pH 7.5)] at room temperature for 12-14 h. The reaction was terminated by addition of 100 µl 1 M DTT. After approx. 25 min, when deprotection of Cys-239 was completed, the mixture was chromatographed on a Sephadex G-25 column (see section 2.4). The concentration of IAEDANS bound to aldolase was determined by absorbance measurements using the molar absorption coefficient at 337 nm of 6000 M$^{-1}$cm$^{-1}$ [21] for cysteine-AEDANS. From the known concentration of AEDANS residues in the protein sample, the contribution of AEDANS to protein absorption at 280 nm was estimated, taking the molar absorption coefficient for AEDANS at 280 nm as 1260 M$^{-1}$cm$^{-1}$ [22]. Assuming additivity of absorption of aldolase intrinsic chromophores and absorption of AEDANS at 280 nm, the aldolase concentration in the analysed sample was calculated. In a separate experiment, the number of exposed SH groups in the aldolase-Cys-289-AEDANS derivative was determined according to the Ellman method.

2.5.3. Reaction of aldolase with IAF at Cys-239. Two millimolar IAF in ethanol (no more than 5% of the final volume) in the presence of 50 mM hexitol-1,6-P$_2$ was added to the aldolase solution in stock buffer [10 mM Tris, 1 mM EDTA (pH 7.5)]. The protein concentration in the reaction mixture was
approx. $1 \times 10^{-5}$ M and that of IAF $4.8 \times 10^{-5}$ M. The reaction was terminated after 2 h by addition of 1 M DTT to the final concentration of 0.01 M. The mixture was chromatographed on a Sephadex G-25 column as described earlier. The number of IAF residues bound per protein molecule was determined using molar absorption coefficients of 48700 M$^{-1}$cm$^{-1}$ and 14000 M$^{-1}$cm$^{-1}$ at 495 nm and 280 nm, respectively [22]. To estimate the specificity of the modification reaction, the number of exposed SH groups and disappearance of fast-reacting thiol were measured in a separate experiment.

2.5.4. Reaction of aldolase with DABMI at Cys-239. Ten millimolar DABMI in DMF and 50 mM hexitol-1,6-P$_2$ were added to the aldolase solution to the final concentration of: protein $1 \times 10^{-5}$ M, DABMI $4 \times 10^{-4}$ M and hexitol-1,6-P$_2$ 0.02 M. The final concentration of DMF was always below 1%. The reaction was terminated with 1 M DTT (final concentration 0.02 M) and then the reaction mixture was passed down a Sephadex G-25 column. The amount of DABMI incorporated to aldolase was calculated by absorbance measurements at 460 nm using a molar absorbance coefficient 24800 M$^{-1}$cm$^{-1}$ [23]. At this wavelength aldolase absorbance is negligible.

To determine the aldolase concentration from the absorbance at 280 nm, the DABMI absorption at this wavelength was measured using a DABMI-DTT derivative as a standard. This derivative was made by incubation of a 100-fold molar excess of DTT with a DABMI solution for 0.5 h at 25°C. It was found that the absorbance of the DABMI derivative at 280 nm is 1.4 times higher than at 460 nm. It was assumed that the aldolase absorption at 280 nm ($A_{280}^{ald}$) equals the absorption observed at 280 nm ($A_{280}^{ob}$) corrected by the factor taken from the DTT-DABMI spectrum:

$$A_{280}^{ald} = A_{280}^{ob} - 1.4 \times A_{460}$$

where $A_{460}$ is the absorption of the aldolase-Cys-239-DABMI at 460 nm.

2.5.5. Preparation of doubly-labeled aldolase. The solution of aldolase-Cys-289-AEDANS was concentrated by ultrafiltration on a PM-10 membrane (Amicon) from approx. $1 \times 10^{-6}$ M to $1.5 \times 10^{-5}$ M. The concentrated sample of aldolase-Cys-289-AEDANS was allowed to react with either DABMI or IAF under conditions that were described above for preparation of the enzyme singly-labeled with those reagents. Again, the aldolase concentration in the doubly-labeled protein was estimated using the corrections for the absorption of labels at 280 nm.

RESULTS AND DISCUSSION

3.1. Preparation and characterization of aldolase-Cys-289-AEDANS.

A crucial step in the specific labeling of aldolase with the fluorescent probes is the preparation of the protein sample blocked with Nbs$_2$ only at the fast-reacting Cys-239. As shown in Table 1 titration of cysteine residues in
**Table 1**

<table>
<thead>
<tr>
<th>ENZYME</th>
<th>Specific activity (%)</th>
<th>Number of exposed -SH groups/aldolase subunit</th>
<th>Number of fast-reacting -SH groups/aldolase subunit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aldolase</td>
<td>100</td>
<td>3.94</td>
<td>0.9</td>
</tr>
<tr>
<td>Aldolase + hexitol-1,6-P₂</td>
<td>&lt;5</td>
<td>1.9</td>
<td>0.9</td>
</tr>
<tr>
<td>Aldolase-Nbs <strong>a</strong></td>
<td>100–105</td>
<td>2.89</td>
<td>0</td>
</tr>
<tr>
<td>Aldolase-Nbs + hexitol-1,6-P₂</td>
<td>&lt;5</td>
<td>0.9</td>
<td>0</td>
</tr>
<tr>
<td>Aldolase-AEDANS <strong>b</strong></td>
<td>95–100</td>
<td>3.24</td>
<td>0.9</td>
</tr>
</tbody>
</table>

*a* Concentration of aldolase derivatives were as follows [in μM]:

- \( c_{\text{ald}} = 1.06 \), \( c_{\text{Nbs}} = 4.56 \).
- \( c_{\text{ald}} = 3.46 \), \( c_{\text{AEDANS}} = 10.0 \).

Aldolase-Nbs derivative showed that only the fast-reacting Cys residue was blocked. The Nbs-modified protein exhibited 100-105% of the native enzyme activity and was stable for at least 24 h at 20°C. Deprotection of the aldolase SH group with DTT yielded 1.05-1.1 Nbs residue released from the aldolase-Nbs derivative (not shown). Thus it is reasonable to believe that the aldolase-Nbs is exclusively labeled at Cys-239 and this preparation is suitable for subsequent modification with IAEDANS. It was found that the amount of 0.72 AEDANS residue incorporated per aldolase subunit nicely corresponds with the disappearance of 0.70 slow-reacting thiol (Table 1). In an independent experiment the time dependence of the AEDANS incorporation into the aldolase-Cys-239-Nbs derivative shows incorporation of 0.86 molecule of

**Table 2**

*Time course of modification of aldolase-Nbs derivative with IAEDANS at 20°C*

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Number of AEDANS residues * incorporated/aldolase-Nbs subunit</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.0</td>
<td>0.38</td>
</tr>
<tr>
<td>7.0</td>
<td>0.53</td>
</tr>
<tr>
<td>12.5</td>
<td>0.78</td>
</tr>
<tr>
<td>14.0</td>
<td>0.86</td>
</tr>
<tr>
<td>16.0</td>
<td>1.00</td>
</tr>
</tbody>
</table>

* The amount of AEDANS residues incorporated into the aldolase molecule was measured as described in Methods. Concentration of reagents were as follows [in μM]:
  - \( c_{\text{ald-Nbs}} = 6.32 \), \( c_{\text{AEDANS}} = 1.5 \times 10^{-5} \). The aldolase-Nbs preparation contained 1.12 of Nbs residue/aldolase subunit.
AEDANS residue per subunit after 14 h of the reaction (Table 2) in the presence of the competitive inhibitor.

The aldolase-AEDANS derivative which was obtained after the deprotection of the aldolase-Nbs-AEDANS sample with DTT (see Section 2.5.2.) was analysed for the number of fast-reacting SH groups which should have reappeared. As shown in Table 1 the aldolase-Cys-289-AEDANS preparation has approximately one fast-reacting SH group available for further modification. This derivative exhibited 95-100% of the initial activity, and no decrease of activity could be detected after 24 h incubation at 20°C. The stoichiometry of the AEDANS incorporation, the SH group analysis and the finding that aldolase-Cys-289-AEDANS is in 100% active, all strongly suggest that, during the modification of aldolase, Cys-72 and Cys-338 were fully protected against reaction with either Nbs₂ or IAEDANS. Thus it is conceivable that aldolase was labeled with IAEDANS only at Cys-289. This derivative showed an absorption band at 337 nm characteristic for the AEDANS residue (Fig. 1A). Excitation of the labeled enzyme at 345 nm resulted in an emission maximum at 495 nm (Fig. 1C).

3.2. Characterization of the aldolase-Cys-239-IAF derivative

The time course of the aldolase reaction with IAF in the presence of the substrate analog is presented in Table 3. After 2 h of the reaction 0.36 IAF residue per aldolase subunit was incorporated to the enzyme.

Titration of the exposed cysteine residues in the aldolase-IAF derivative showed that only the fast-reacting Cys-239 residue was modified and we found that about 40% of fast-reacting groups disappeared (not shown). This derivative exhibited 100-105% of the initial activity, and no decrease in activity could be detected after 24 h incubation at 20°C.
Fig. 2. A, the absorption spectrum of aldolase (3.8 \times 10^{-6} \text{ M}) labeled with IAF (0.38 IAF residue per aldolase subunit) in the same buffer as in Fig. 1. B, the absorption spectrum of aldolase (4.82 \times 10^{-6} \text{ M}) labeled with DABMI (0.44 DABMI residue per aldolase subunit) in the same buffer.
Table 3

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Number of IAF residues* incorporated/aldolase-subunit</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>0.14</td>
</tr>
<tr>
<td>1.0</td>
<td>0.24</td>
</tr>
<tr>
<td>2.0</td>
<td>0.36</td>
</tr>
<tr>
<td>4.0</td>
<td>0.41</td>
</tr>
<tr>
<td>5.0</td>
<td>0.43</td>
</tr>
<tr>
<td>7.0</td>
<td>0.60</td>
</tr>
</tbody>
</table>

* Concentration of reagents were as follows [in μM]: \( c_{IAF} = 13.0; \) \( c_{IAF} = 58.0; \)
\( c_{re} = 1.0; \) \( p_2 = 1.0000. \)

The absorption spectrum of the aldolase-Cys-239-IAF derivative has a maximum at 495 nm (Fig. 2A). As expected, the maximum of the fluorescence excitation spectrum was found at the same wavelength (not shown). The emission band appeared at 520 nm (not shown).

3.3. Characterization of the aldolase-Cys-239-DABMI derivative

The time course of the aldolase reaction with DABMI in the presence of substrate analog is presented in Table 4. As one may note the modification reaction of aldolase with DABMI is similar to that with IAF. Also the titration

Table 4

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Number of DABMI residues* incorporated/aldolase-subunit</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>0.20</td>
</tr>
<tr>
<td>1.0</td>
<td>0.28</td>
</tr>
<tr>
<td>2.0</td>
<td>0.33</td>
</tr>
<tr>
<td>4.0</td>
<td>0.42</td>
</tr>
<tr>
<td>7.0</td>
<td>0.58</td>
</tr>
</tbody>
</table>

* Concentration of reagents were as follows [in μM]: \( c_{DABMI} = 11.0; \) \( c_{DABMI} = 0.400; \)
\( c_{re} = 1.0; \) \( p_2 = 1.0000. \)

of fast-reacting cysteines in aldolase-DABMI showed that only Cys-239 was modified (not shown). The labeled aldolase exhibited 97-102% of the initial activity and was stable during the 24 h incubation at room temperature.

The aldolase-Cys-239-DABMI derivative showed an absorption band at 280 nm and a plateau between 350-500 nm (Fig. 2B). DABMI and its protein derivatives do not fluoresce but can be used as an energy acceptor in electronic excitation energy transfer experiments [23].
3.4. Characterization of doubly-labeled aldolase

As indicated in Methods, the aldolase-Cys-289-AEDANS derivative was used as a substrate for preparation of doubly-labeled protein samples. This compound contained approximately one fast-reacting SH group available for

![Absorbance vs Wavelength](image)

Fig. 3. The absorption spectrum of doubly labeled aldolase in the same buffer as in Fig. 1. A, the enzyme ($4.82 \times 10^{-6}$ M) contained 0.70 AEDANS and 0.45 DABMI residues incorporated/aldolase subunit. B, the enzyme ($5.33 \times 10^{-6}$ M) contained 0.73 AEDANS and 0.38 IAF residues incorporated/aldolase subunit.
further modification (see 3.1 and Table 1). We found that the time course of modification of aldolase-Cys-289-AEDANS with IAF or DABMI is the same as in the case of the modification of native aldolase with these chromophores (not shown), suggesting that the modification reaction occurs at the fastest-reacting Cys-239. The absorption spectrum of aldolase-Cys-289-AEDANS-Cys-239-DABMI showed bands characteristic of both singly labeled preparations (Fig. 3A) with maxima at 337 nm corresponding to AEDANS absorption and maximum at 450 nm attributable to DABMI absorption. Aldolase-Cys-289-AEDANS-Cys-239-IAF showed two absorption bands at 337 nm and 495 nm, corresponding to IAF and AEDANS absorption maxima, respectively (Fig. 3B). The preparations of doubly-labeled aldolase exhibited 95-100% of the initial enzyme activity. This allows us to assume that the above fluorescent probes do not affect substantially the aldolase structure and the active site region and can be applied for protein dynamics studies.

The preparations of aldolase modified specifically with IAF or DABMI at Cys-239 and with IAEDANS at Cys-289 were used as fluorescent donor-acceptor pairs. Preliminary aldolase topography studies showed energy transfer between the two chromophores of the enzyme (in preparation).

We thank Dr Tomasz Heyduk for useful discussion.

REFERENCES


