CATALYTICALLY ACTIVE AZOALDOLASE: IDENTIFICATION OF CYSTEINYL RESIDUES INVOLVED IN THE DIAZOTHIOETHER CHROMOPHORES FORMATION

E. BALESTRERI*, M. BRACALONI, G. MONTAGNOLI and R. A. FELICIOLI

*C.N.R. – Laboratorio per lo Studio delle Proprietà Fisiche di Biomolecole e Cellule, Via F. Buonarroti 9, – 56100 Pisa, Italy and *Laboratorio di Chimica Biologica Facoltà di Scienze M.F.N., Via A. Volta 4, – 56100 Pisa, Italy*

Received 9 June 1975

1. Introduction

The procedure for the preparation of a catalytically active azoaldolase has been recently described. It mainly consists in coupling at pH 7.2 the rabbit muscle aldolase, adsorbed on phosphocellulose, with diazotized *p*-aminobenzoate. The homogeneously labeled active azoaldolase is then specifically eluted from the phosphocellulose by the use of a 2.5 mM solution of the substrate fructose 1,6-bisphosphate. The chemical modification is very specific: it leads to the synthesis of 8 diazothioether chromophores, equally distributed among the four subunits [1].

It is well known that rabbit muscle aldolase contains 32 cysteinyl residues per molecule (i.e. 8 per subunit) and on the basis of both their reactivity towards a large series of sulphydryl reactants and the protection exerted by the substrate or its analogues, they have been classified in 'buried' (cysteine residues no. 134, 149, 177 and 199), 'exposed' (cysteine residues no. 237 and 287) and 'protected' (cysteine residues no. 72 and 336 [2]. The spatial localization in connection with the active site and the role in the proposed mechanism of action of aldolase, have also been tentatively indicated: in particular it has been shown that the mild oxidation of cysteinyl residues 72 and 336 to form an intrachain S–S bridge brings about complete inactivation of the enzyme [3,4]. We report herein the results of experiments carried out with the aim of identifying the modified cysteinyl residues of azoaldolase.

2. Materials and methods

FDP aldolase (EC 4.1.2.13) was purified from rabbit muscle following the procedure of Lai [5] and crystallized four to five times at 54% saturation ammonium sulfate solution buffered at pH 7.2. Azoaldolase was prepared following the procedure described by Felicioli et al. [1]. Enzyme assays were carried out according to Racker [6]. The protein concentration was spectrophotometrically determined using a molar absorption coefficient at 280 nm of 150 000 $M^{-1} cm^{-1}$ [7] for native aldolase. For each azoaldolase preparation the molar absorption coefficient at 280 nm was established by means of the ratio of the absorbances at 330 nm and at 280 nm and using the previously published calibration curve [1]. The determination of the free SH was carried out in 100 mM Tris–Cl buffer pH 8.0 using 9.10^{-7} M enzyme solution and 4 mM DTNB in the presence of 2 mM EDTA according to Ellman [8].

Oxidation of both native aldolase and azoaldolase was performed by incubating $5 \cdot 10^{-6}$ M protein solution with 0.07 mM *o*-phenanthroline and cupric ion (1/1 ratio) in 50 mM Tris–Cl buffer pH 8.5 according to Lai [4].

At different time intervals aliquots of the reaction mixture were withdrawn and separately assayed for enzyme activity and free SH content.

The time dependent hydrolysis of the diazothioether bonds under the experimental conditions needed for *o*-phenanthroline oxidation has been followed by recording the decrease in absorbance at 330 nm. Quantitative estimations have been made by using a molar absorbtivity of 15 700 $M^{-1} cm^{-1}$ determined

for the model compound *p*-carboxybenzenediazonium derivative of *N*-acetylcysteine [9].

3. Results

Table 1 summarizes the results of experiments of free SH titration in both native rabbit muscle aldolase and azoaldolase, in the presence or in the absence of SDS. The reported data, taken together with analogous evidences obtained with mercurials, indicate that azoaldolase mainly differs from native aldolase because of 8 exposed cysteinyl residues. These findings completely agree with the evidence previously collected by both spectroscopy and aminoacid analysis on 8 diazothioether chromophores per molecule [1].

Fig.1 shows the time course of free SH titration in both native rabbit muscle aldolase and azoaldolase. As it can be seen the difference of 8 cysteinyl residues between the two aldolases is reached at long (25 min) reaction times even though values approaching 8 are calculable also at very short reaction times (i.e., at 5 min the observed difference in titrable SH groups is 6). The time course of titration of 'fast reacting' SH residues of both aldolases has not been recorded, however the reported results clearly indicate that no differences are observable as far as the reactivity of

Table 1
Free SH groups in rabbit muscle aldolase and azoaldolase

State	Rabbit muscle aldolase (a)	Azoaldolase (b)	SH (a–b)
Native	15.8	8	7.8
Denaturated with 2% SDS	32	24	8

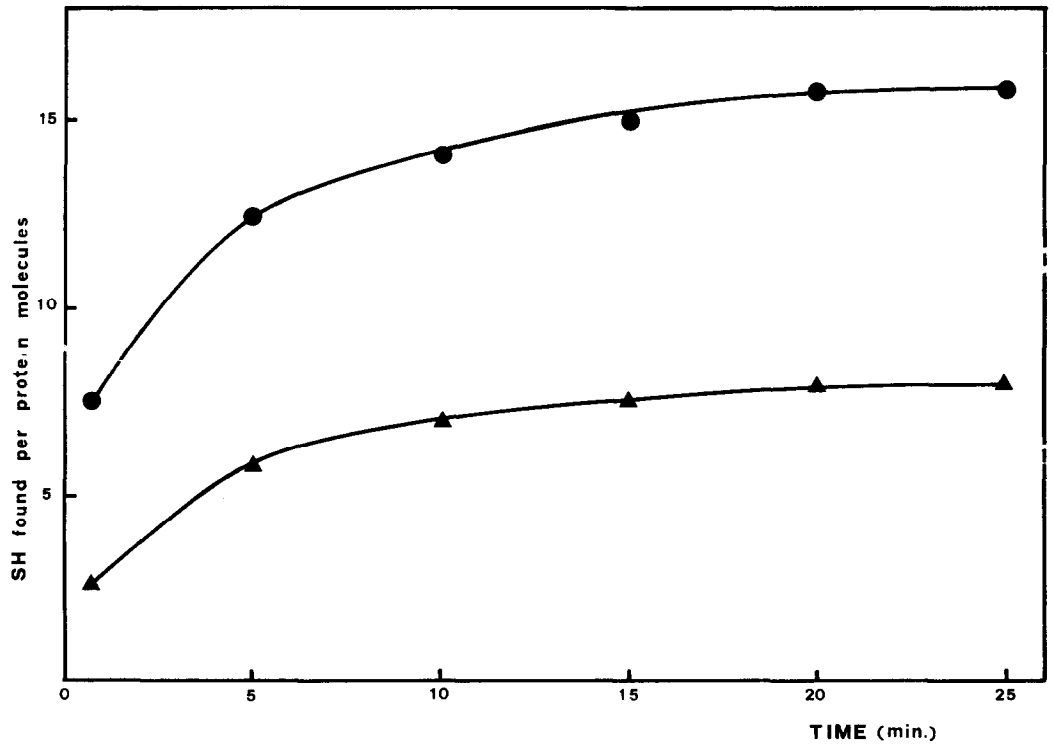

Fig. 1. Time course of the titration of free SH in both native rabbit muscle aldolase (●—●) and azoaldolase (▲—▲). The experiment was carried out in 100 mM Tris–Cl buffer pH 8.0 using $9 \cdot 10^{-7}$ M enzyme solution and 4 mM DTNB in the presence of 2 mM EDTA according to Ellman [8].

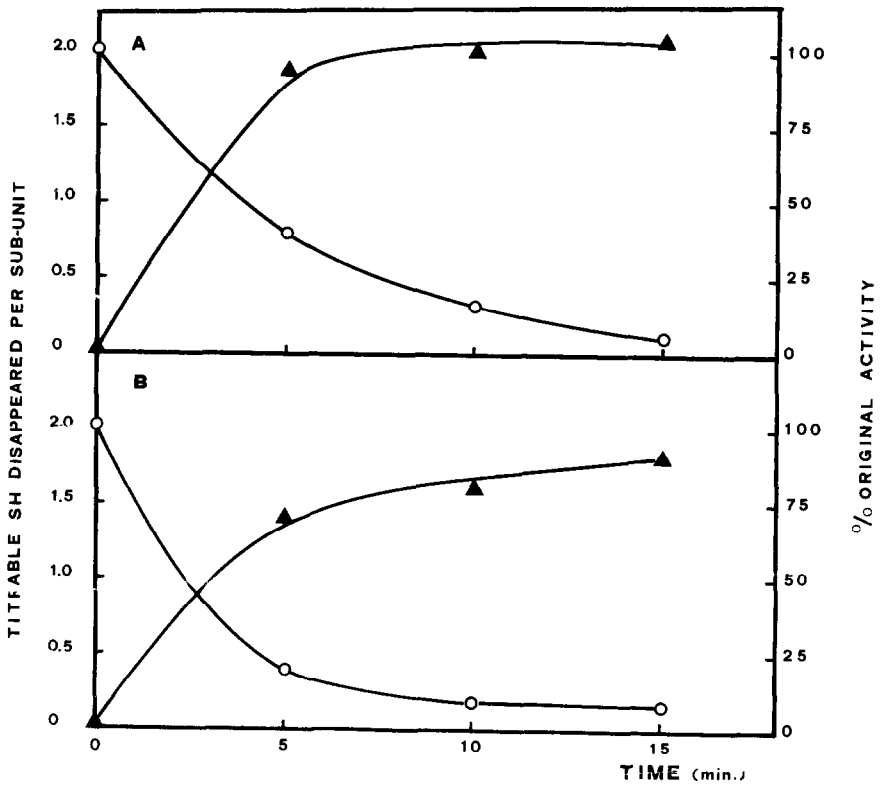

Fig. 2. Activity decrease and intrachain S–S bridge formation upon oxidation by cupric-*o*-phenanthroline complex of rabbit muscle aldolase (A) and of azoaldolase (B). Protein solution ($5 \cdot 10^{-6}$ M) were incubated at room temperature and pH 8.5 with 0.07 mM *o*-phenanthroline and cupric ion (1:1 ratio). At the reported time intervals samples were withdrawn and separately assayed for residual enzyme activity (o—o) and for disappearance of free SH group (▲—▲).

'slow reacting' SH groups is concerned [10].

The dependence of azoaldolase catalytical activity on the oxidation by *o*-phenanthroline is reported in fig.2 (B). For comparison the inactivation of native rabbit muscle aldolase fig.2 (A) is also reported. We have found that in both cases the inactivation observed is reversed on addition of excess of free sulphydryl groups (by addition of dithiothreitiol) as would have occurred if the inactivation had been dependent on S–S bridge formation.

4. Discussion

The reversible inactivation of azoaldolase through SH oxidation, without change in the number of diazothioether bonds indicates that cysteinyl residues number 72 and 336 are accessible to *o*-phenanthroline oxidation and therefore must have remained unmodified during the coupling reaction.

Furthermore the observed unchanged catalytic activity of the azoaldolase is in agreement with the assignment of chemical modification to cysteinyl residues 237 and 287 and may be explained in the light of the previously reported experimental evidences that neither of them is essential for the catalytic process, even though cysteinyl residue 287 has been classified as 'contributing' to the protein conformation [2].

As in the case of all the reported amino acid analyses of azoproteins containing azo derivative of tyrosine, histidine and lysine [11] the intermediate initially formed cannot be identified after the acidic hydrolysis. Recently Chan and Anderson [12] have reported the presence of the thioether in the amino acid analysis of diazo coupled yeast alcohol dehydrogenase. In the

case of azoaldolase prepared in the dark the nature of the cysteinyl derivative has been identified by comparison of the absorbance spectra and of the photochemical behaviour of azoaldolase and of the model compound diazothioether of *N*-acetyl-cysteine [1,9].

Finally the reported data provide some conclusions for the interaction between aldolase and phosphocellulose at neutral pH which suggest affinity chromatography rather than an affinity elution mechanism [13]. By analogy with the observed protection exerted by substrates or analogoues toward chemical modification [2,14,15], 'protection' by phosphocellulose of some of the amino acid residues essential to the catalytic activity can be postulated: among them at least cysteinyl residues 72 and 336 [4]. It is noteworthy that the azoaldolase prepared by coupling in solution at pH 8.8 is catalytically inactive and bears 14 modified cysteinyl residues per molecule of enzyme [1].

References

[1] Felicioli, R., Nannicini, L., Balestreri, E. and Montagnoli, G. (1975) Eur. J. Biochem. 51, 467–473.
[2] Steinman, H. M. and Richards, F. M. (1970) Biochemistry 9, 4360–4371.
[3] Kobashi, K. and Horecker, B. L. (1976) Arch. Biochem. Biophys. 121, 178–186.
[4] Lay, C. Y., Chen, C., Smith, J. D. and Horecker, B. L. (1971) Biochem. Biophys. Res. Commun. 45, 1497–1505.
[5] Lay, C. Y. (1968) Arch. Biochem. Biophys. 128, 202–211.
[6] Racker, E. (1947) J. Biol. Chem. 167, 843–854.
[7] Donovan, J. W. (1964) Biochemistry 3, 67–73.
[8] Ellman, G. L. (1959) Arch. Biochem. Biophys. 82, 70–77.
[9] Montagnoli, G., Monti, S. and Nannicini, L. (1975) Gazz. Chim. Ital., in the press.
[10] Eagles, P. A. M., Johnson, L. N., Johnson, M. A., McMurray, C. H. and Gutfreund, H. (1969) J. Mol. Biol. 45, 533–544.
[11] Riordan, J. F. and Vallee, B. L. (1972) Methods Enzymol. 25B, 521–531.
[12] Chan, J. K. and Anderson, B. M. (1975) J. Biol. Chem. 250, 67–72.
[13] Von Der Haar, F. (1974) Methods Enzymol. 34B, 163–171.
[14] Anderson, P. J. and Perham, R. N. (1970) Biochem. J. 117, 291–298.
[15] Szajàni, B., Sajgò, E. Biszku, E., Friedrich, P. and Szabolcsi, G. (1970) Eur. J. Biochem. 15, 171–178.