EVIDENCE FOR AN INTERPROTOMERIAL ACTIVE SITE IN D-GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE

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1. Introduction

Rabbit muscle D-glyceraldehyde-3-phosphate dehydrogenase (GAPD) consists of four identical polypeptide chains [1], each accomodating one molecule of NAD^+ as a coenzyme. In its amino acid sequence it is similar to the pig muscle enzyme, whose primary structure has been established [1]. It has been shown [2] that the sulfhydryl group of the cystein residue at position 149* is essential for the catalytic activity of the enzyme. Upon removal of NAD^+ this cystein residue can be selectively dinitrophenylated with concomitant loss of enzymatic activity [3,4]. The unique reactivity of this sulfhydryl was used [5] as an anchoring point for the bifunctional labeling reagent 1, 5-difluoro 2,4-dinitrobenzene (F_2 DNB)**, thus introducing crosslinks at the NAD^+ binding site of the enzyme. One type of crosslink was formed between the sulfhydryl group of Cys-149 and the ϵ-amino group of Lys-183 [5]. Another type of crosslink connected Cys-149 and Cys-153 [6]. It was shown that the crosslinks were formed within the tetrameric molecule, as there was no change in its sedimentation coefficient as a result of the labeling. The possibility was raised [5] that the crosslinked Cys and Lys residues might originate from two adjacent protomers within the tetrameric molecule. We wish to report here direct chemical evidence for the suggestion that two adjacent protomers provide functional groups to the NAD^+ binding site, i.e., that GAPD has an interprotomerial active site.

2. Materials and methods

Rabbit muscle apo-GAPD (from Sigma) was prepared, characterized and labeled with F_2 DNB by the procedures described earlier [3–5]. Sedimentation velocity studies were carried out, with either the unlabeled or with the labeled apoenzyme, following dialysis against 20 volumes of 6 M guanidine hydrochloride and reaction with *N*-ethylmaleimide according to the procedure described by Harrington and Karr [7]. Sedimentation coefficients were measured at 20°C in a Spinco model E analytical ultracentrifuge with a rotor speed of 52 640 rev/min. Sedimentation coefficient values (s°_{25}) were calculated by introducing viscosity and density corrections [8]. Acrylamide gel electrophoresis was carried out in the presence of sodium dodecyl sulfate (0.1%) on gels containing 5% acrylamide [9]. The molecular weight scale was calibrated with pepsin (35 000), bovine serum albumin (69 000) and myosin (200 000).

3. Results and discussion

Apo-GAPD was reacted with stoichiometric amounts of F_2 DNB as described elsewhere [5]. An aliquot of the reaction mixture was removed, digested

* Residues numbers refer to the sequence of the pig muscle enzyme [1].

** *Abbreviations*: F_2 DNB: 1,5-difluoro 2,4-dinitrobenzene. GAPD: D-glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12)

with pepsin, and shown to contain the Cys-149 to Lys-183 crosslink (for the procedure see [5]). The labeled enzyme was then dialyzed against 6 M guanidine hydrochloride and reacted with *N*-ethylmaleimide. A control sample of the apoenzyme was subjected to the same procedure, omitting only the labeling reagent.

Sedimentation velocity experiments were carried out on these two samples, in an attempt to find out whether the formation of the crosslinks prevents the dissociation of the tetrameric enzyme (mol. wt. 144 000) [1,7] into its monomers (mol. wt. 36 000 daltons [1,7]). It was found that in 6 M guanidine hydrochloride the unlabeled apoenzyme migrated as a single symmetrical boundary. Under identical experimental conditions, the crosslinked apoenzyme did not produce a symmetrical boundary, suggesting heterogeneity in the sedimenting species.

Prompted by this observation, we extended the sedimentation velocity measurements of both labeled and unlabeled apoenzyme solutions in 6 M guanidine hydrochloride to cover a protein concentration range of 1.5–10 mg/ml. As seen in fig.1, sedimentation coefficients of unlabeled apo-GAPD (the lower curve) extrapolate to $s^{\circ}_{25} = 0.75$ S, in good agreement with the value of $s^{\circ}_{25} = 0.76$ S calculated for this system [8] on the basis of measurements performed by Harrington and Karr [7]. Sedimentation coefficients of the labeled apoenzyme (upper curve) extrapolate

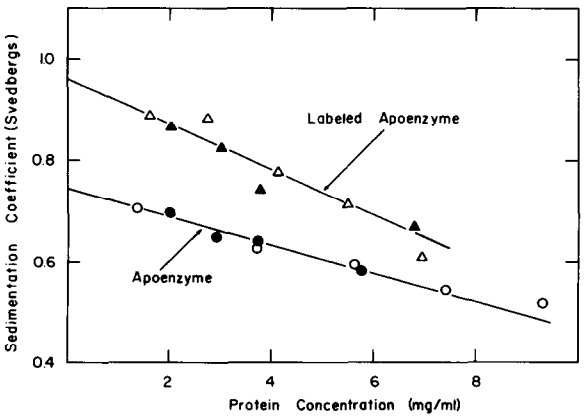

Fig.1. Effect of the crosslinking of apo-GAPD on its dissociation in 6 M guanidine hydrochloride. Open and filled symbols represent independent sets of experiments.

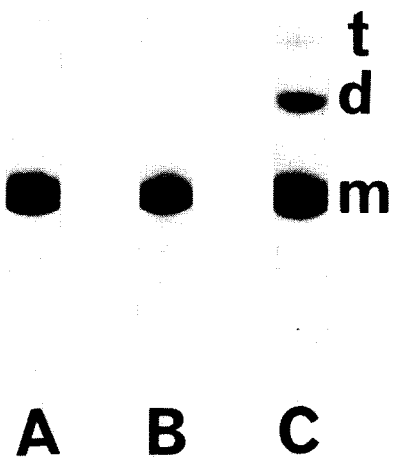

Fig.2. Comparison between unlabeled, monofunctionally labeled and bifunctionally labeled apoenzyme gel electrophoresis in the presence of sodium dodecyl sulfate. (A) unlabeled apo-GAPD; (B) monofunctionally labeled apo-GAPD prepared by reaction with 1-fluoro-2,4 dinitrobenzene; (c) bifunctionally labeled apo-GAPD prepared by reaction with F_2 DNB; m, monomers; d, dimers; t, tetramers, with observed mol. wts of 33 000, 72 000, 123 000 respectively.

to a much higher value, $s^{\circ}_{25} = 0.96$ S and exhibit a different concentration dependence.

Had the system under study been homogeneous, the value of $s^{\circ}_{25} = 0.96$ S would roughly correspond to a polypeptide chain of mol. wt close to 65 000 (cf, table II in [8]). In the present case, where heterogeneity of the system was implied by the shape of its sedimentation boundary, we postulated that the higher value of s°_{25} = is due to the presence of monomers, dimers and perhaps higher aggregates. This assumption was verified by acrylamide gel electrophoresis. As seen in fig.2, both unlabeled apo-GAPD and an apoenzyme preparation which had been labeled [3,4] by a monofunctional dinitrophenylating reagent (1-fluoro-2,4-dinitrobenzene) migrate as a single band. However, the apoenzyme which had been crosslinked with F_2 DNB contained two major bands with mol. wts of 33 000, 72 000 and a minor band with a mol. wt of 12 3000. Though no quantitative estimate of the relative amounts of these three components was attempted, it is clear that the monomer prevails, indicating that only in some of the molecules was dissociation impeded as a result of interprotomerial crosslinking.

The fact that a considerable amount of the protein was dissociated into monomers is in accordance with our finding ([5] and unpublished results) that the yield of the two Cys-Lys crosslinked peptides which were isolated is relatively low (∿ 20% in the peptic digest mixture), that other crosslinks which were identified are intraprotomerial [6] (fig.1) and that the bifunctional reagent, once anchored to Cys-149, may undergo hydrolysis of its second C-F bond and thus end up as monovalent label.

By passage through a molecular sieve (Sephadex G-150) under conditions which preserve the protein in the dissociated state (eluent: 8 M urea solution containing 1 M propionic acid), we were able to separate the mono-functionally labeled monomers from the crosslinked dimers and to show by spectral and chemical analysis that the dimers do indeed contain the Cys-Lys crosslink.

Rossmann et al. [10,11] have recently described the structure of lobster GAPD obtained by X-ray crystallography at 3Å resolution. They found that in the crystalline holoenzyme, NAD^+ binds to the protein close to the subunit interfaces and that Lys 183 of one protomer interacts with the pyrophosphate of the NAD^+ in the adjacent protomer. This puts the ϵ-amino group of Lys-183 in protomer (a) at a distance of 12 Å from the sulfhydryl group of Cys-149 in protomer (a') [11]. Our previous studies suggested [5] that in the rabbit muscle apoenzyme these two functional groups may approach each other to a distance of ∿6 Å, as they can be crosslinked with F_2DNB (fig.3). Such distance could account for the observation of Polgar and Harris [12] that an *S*-acetyl group on Cys-149 can be made to migrate to the ϵ-amino group of Lys-183 upon raising the pH of the medium. The results presented here provide direct physicochemical evidence to the suggestion that the crosslinked -SH and NH_2 groups originate from two adjacent protomers (fig.3). The difference in distance between them (∿6 Å in the apoenzyme vs. 12 Å in the holoenzyme) may be due to one or more of the following reasons: a) the conformation of the NAD^+ site may be altered upon removal of the cofactor (several physicochemical measurements indicate considerable structural differences between the apoenzyme and the holoenzyme [13–19]; b)

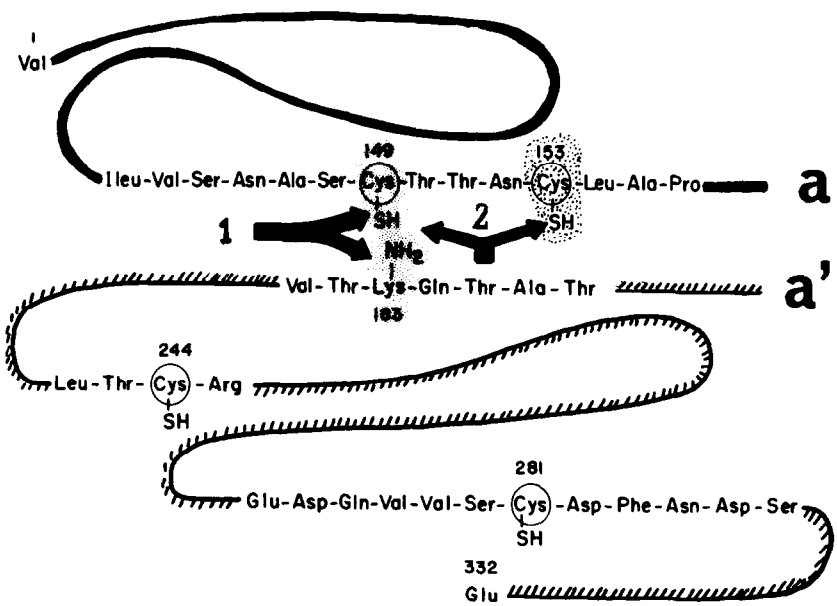

Fig.3. Proposed structure for the active site (shaded area) of apo-GAPD. Arrows 1 and 2 indicate the crosslinks established by labeling with F_2 DNB [5,6], which suggest proximity of the three indicated functional groups in the three dimensional structure of the apo-enzyme. Note that the Cys-149 and Lys-183 residues are suggested to be provided by two adjacent protomers (a and a') while Cys-149 and Cys-153 which are also crosslinked by F_2DNB [6] are provided by the same protomer.

the very anchoring of the bifunctional label onto Cys-149 may induce a conformational change bringing the -SH and -NH_2 groups closer together; c) the structure of the protein in solution may differ from that of the crystalline enzyme.

The above mentioned interprotomerial relationships may play an important role in the co-operative binding of NAD^+ to the protein [20–24] and in the phenomenon of half-of-the-sites reactivity observed during reaction of the enzyme with certain alkylating or acylating reagents [25–29].

Studying the dissociation of other multisubunit enzymes, crosslinked at their active sites, may reveal that GAPD is not unique in having interprotomerial active sites. In fact, such sites may have evolved to meet with definite physiological assignments.

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