INVESTIGATION OF THE QUATERNARY STRUCTURE OF BEEF LIVER GLUTAMATE DEHYDROGENASE WITH BIFUNCTIONAL REAGENTS

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SUMMARY

The six identical polypeptide chains of the smallest enzymatically active unit of beef liver glutamate dehydrogenase are shown to be arranged in two trimers. Cross-linking with bifunctional reagents of varying chain length and subsequent SDS polyacrylamide gel electrophoresis of the protein shows main bands corresponding to molecular weights of 168,000 and 336,000 daltons which is three times and six times, respectively, the molecular weight of the polypeptide chain (56,000 daltons). This finding supports a model for the quaternary structure of the glutamate dehydrogenase proposed by Reisler and Eisenberg (Biopolymers 9, 877 (1970)).

The smallest enzymatically active unit of beef liver glutamate dehydrogenase is a hexamer composed of six identical polypeptide chains (1,2). Based on electron microscopic studies (3,4) and the X-ray small-angle scattering measurements (5) a model has been proposed which contains the six polypeptide chains arranged in two layers of three (6,7). Interpretation of the electron micrographs is difficult and since they might not even render a picture of the arrangement of the polypeptide chain of the native enzyme in solution, direct proof for the proposed model has not yet been obtained. Knowledge of the exact structure of the hexamer is pertinent to the interpretation of observations from kinetic studies (8), ligand binding experiments (9-11) and chemical modifications of the enzyme (12-14) which have shown that certain functional groups, as well as the ligand binding sites in the active center of the hexamer, react in two sets of three (reviewed in (15)). For example, different dissociation constants for each three reduced coenzyme binding sites

have been obtained (11) and for NAD^+ only three binding sites can be determined in a reasonable concentration range of the coenzyme (10).

If the proposed model is right there should exist at least two different types of binding domains between the polypeptide chains of a hexamer, one between the chains within the layer of three the other between the two layers. Different parts of the polypeptide chain should form these contacts and therefore differences in the functional groups involved could occur. Using bifunctional reagents, we present evidence in this paper that such differences indeed exist. By cross-linking the polypeptide chains with diimidates of various chain lengths, we could show that glutamate dehydrogenase polypeptide chains can be covalently linked together predominantly in groups of three. Only at very high concentrations of the bifunctional reagent and at high chain length, i.e. with suberimidate containing an eight carbon chain, all six or even more polypeptide chains could be linked together.

EXPERIMENTAL

Materials: Crystalline glutamate dehydrogenase, NADH, α-ketoglutarate were obtained from Boehringer Mannheim GmbH (Mannheim, Germany). Triethanolamine, analytical grade, was purchased from E. Merck (Darmstadt, Germany). The diimidates were synthesized according to the method of McElvain (16) from the corresponding dinitriles. The dinitriles were products of Fluka, Buchs, Switzerland (adipinic-, pimelinic-, and subericdinitril), E. Merck, Darmstadt, Germany (malonic dinitril) and EGA-Chemie KG, Steinheim, Germany (succinic dinitril). All other chemicals were of the highest grade commercially available.

Methods: Cross-linking with diimidates:
Cross-linking was performed as described by Davies and Stark (17). Crystalline glutamate dehydrogenase was dialyzed overnight against three changes of 0.067 M phosphate buffer, pH 7.6, and diluted with about thirty times its volume of 0.2 M triethanolamine buffer, pH 8.1, to a final protein concentration of 0.5 mg/ml. The protein was incubated with the imidate for about three hours at room temperature.

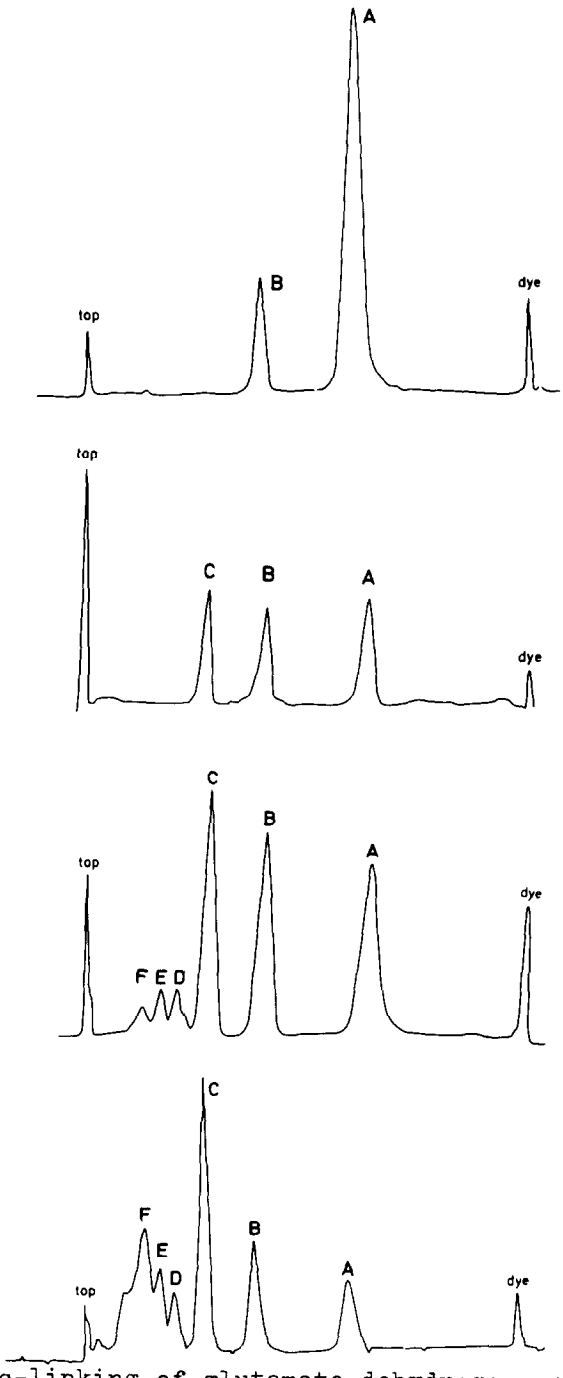

Fig. 1: Cross-linking of glutamate dehydrogenase with diimidates of varying chain length. SDS polyacrylamide gels were scanned at 550 nm. Cross-linking was performed with 0.5 mg/ml glutamate dehydrogenase in 0.2 M triethanolamin buffer, pH 8.5, with 0.2 mg/ml (from top to bottom) malonimidate, adipimidate, pimilimidate and suberimidate. Incubation time was three hours at room temperature. The peaks represent the glutamate dehydrogenase polypeptide chain monomer (A), dimer (B), trimer (C), tetramer (D), pentamer (E), and hexamer (F).

Polyacrylamide gel electrophoresis in sodium dodecyl sulfate: Electrophoresis was performed by a method similar to that described by Davies and Stark (17). SDS and ß-mercaptoethanol were added to the protein samples at final concentrations of 1 %. After incubating for 1 min, 50 - 100 µl of the mixture, containing about 50 µg of protein, were applied to a 5 % polyacrylamide gel containing 0.135 % methylene bisacrylamide. Electrophoresis buffer was 0.1 M borate, 0.1 M acetate, 0.1 % SDS, pH 8.5. Electrophoresis was done at 7 mA per tube for about 1.5 hr.

The gels were stained by incubation for 6 hr with 0.07 % Coomassie blue in 45 % methanol, 10 % acetic acid, 45 % water. Destaining was accomplished by overnight shaking of the gels in 30 % methanol, 10 % acetic acid, 60 % water in the presence of a small amount of an ion-exchange resin. The gels were scanned at 550 nm with Beckman Acta III recording spectrophotometer.

Measurement of the enzymatic activity: The enzymatic activity of the glutamate dehydrogenase was measured as described elsewhere (18). In experiments where cross-linking was performed in the presence of NADH and α-ketoglutarate, the substrate and coenzyme were removed prior to the assay by extensive dialysis against 0.067 M phosphate buffer, pH 7.6.

Ultracentrifugation: To compare the sedimentation coefficient of the native and the cross-linked enzyme, the preparations were centrifuged with a Spinco model-E analytical ultracentrifuge (Beckman Instruments, München, Germany). The enzyme was concentrated to about 3 mg/ml with dry Sephadex G 100 and subsequently dialyzed against 0.067 M phosphate buffer, pH 7.6.

RESULTS

Glutamate dehydrogenase was incubated with diimidates of various chain lengths. Subsequent electrophoresis on SDS-polyacrylamide gels showed that cross-linking of the polypeptide chains occured to various extents (Fig. 1). Dimethyl malonimidate reacts only poorly, i.e. the uncross-linked polypeptide

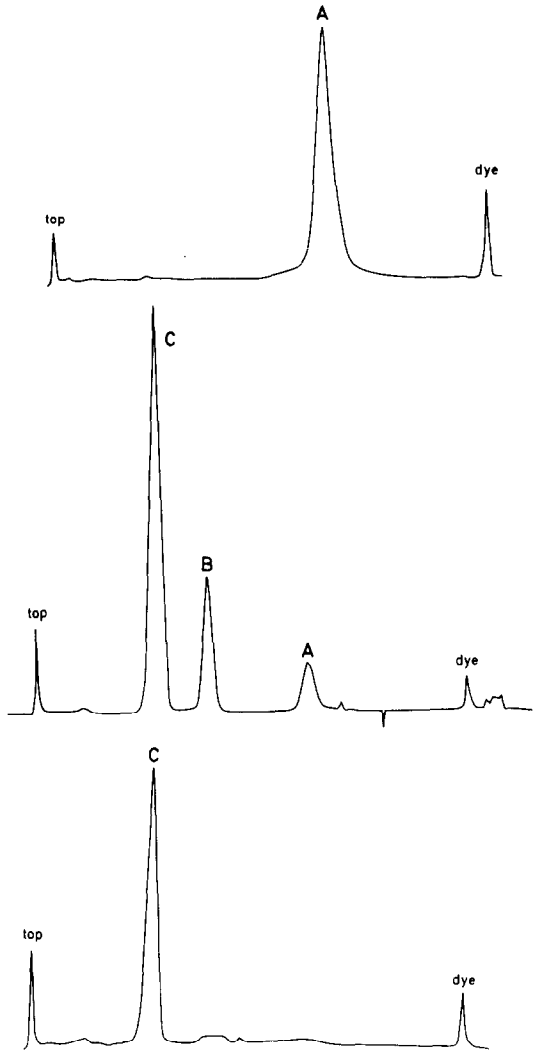

Fig. 2: Cross-linking of glutamate dehydrogenase with dimethyl adipimidate. The SDS polyacrylamide gels were scanned at 550 nm. Cross-linking was performed with 1 mg/ml (midle) and 3 mg/ml (bottom) adipimidate. Control (top) was the gel of the uncross-linked enzyme. The other conditions were as in Fig. 1. The peaks represent the glutamate dehydrogenase monomer (A), dimer (B), trimer (C), and hexamer (D).

chain predominates (Fig. 1, top). The enzyme reacted with suberimidate, on the other hand, yields a gel pattern with many bands after electrophoresis in SDS (Fig. 1, bottom). The lowest molecular weight band represents the polypeptide chain of 56,000 daltons molecular weight, the other bands are multiples of this (19). At least seven bands are visible indicating that

Table 1

Residual activity of glutamate dehydrogenase after cross-linking

Imidate (1 mg/ml)	Residual activity (%)	Uncross-linked protein (%)
Malonimidate	76.5	85
Succinimidate	84	81
Adipimidate	76	10
(without protection by NADH/α-ketoglutarate:)	(26)	(11)
Pimilimidate	41	8
Suberimidate	39.5	12
no imidate	100	100

Cross-linking was performed at a protein concentration of 0.4 mg/ml in the presence of 1 mM NADH and 1 mM α-ketoglutarate. For other conditions see 'Experimental'. The values for the uncross-linked protein have been calculated on the basis of the integrated scann peaks of the SDS gel electrophoresis, % monomer/total protein.

besides the monomer, dimers, trimers, tetramers, pentamers, hexamers, heptamers and possibly larger particles have been formed. The third band representing the trimer predominates. The most striking result was obtained with adipimidate because here the cross-linking seems to stop with the trimer (Fig. 1). Almost no larger particles are present.

In Fig. 2 the concentration of the reagent is varied rather than the chain length. It shows that with a large excess of adipimidate only traces of the hexamer are formed. Almost all the protein has been cross-linked to trimers.

No difference of the band pattern is obtained if one performes the reaction in the presence of NADH and -ketoglutarate or NADH and GTP. NADH/α-ketoglutarate (1 mM) protect the enzyme against inactivation by the imidate, but even without protection it is not completely inactivated (Tab. 1). Especially with adipimidate the percentage of residual activity by far exceeds the percentage of uncross-linked enzyme. So the cross-linked poly-

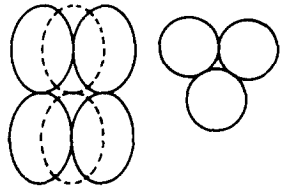

Fig. 3: Model of the beef liver glutamate dehydrogenase, arranging the six polypeptide chains of the smallest enzymatically active unit in two layers of three, according to (6,7). This model implies different binding domains between and within the layers.

peptide chains have to be at least partially active. Table 1 shows the residual activity after cross-linking with the various reagents.

The cross-linked enzyme looses its ability to associate linearly to higher associated particles. Under otherwise identical conditions, the sedimentation coefficient $S_{20,w}$ of the native enzyme is 22.3 S, the one of the cross-linked enzyme 12.7 S (2.6 mg/ml). The latter corresponds to the glutamate dehydrogenase hexamer (336,000 molecular weight).

DISCUSSION

The experiments described in this paper clearly show that the smallest active unit of glutamate dehydrogenase, i.e. the hexamer, is composed of two sets of three polypeptide chains. The chemical reactivity of the binding domains within the set of three towards the cross-linking reagent is similar if not identical. The binding domain between one group of three and the next reacts much slower and only after most of the polypeptide chains are incorporated into trimers. This result is most obvious with adipimidate. One has to be careful not to draw conclusions concerning the distance between the different entities. The different results obtained with diimidates of various chain length may represent different reactivities of the reagent as well as different accessibilities and reactivities of the functional groups of the protein.

The results reported here support the model of the glutamate

dehydrogenase hexamer proposed by Reisler and Eisenberg (6,7) on the basis of X-ray small angle scattering measurment (5) and electron micrographs (3,4). The latter had shown that the enzyme molecule had a triangular appearance and concluded that the six polypeptide chains are arranged in two layers of three (Fig. 3). This model implies different binding domains between and within the layers. Our experiments suggest that this arrangement indeed exists under native conditions of the enzyme in solution.

The cross-linked enzyme partly retains its activity. This indicates that functional groups of the active site of the protein are not involved in the cross-linking reaction. It is tempting to separate the cross-linked trimers from each other and to check if they are still active. Stabilized by the bifunctional reagent, the trimer might turn out to be the smallest active unit and not the hexamer. Attempts to dissociate the cross-linked enzyme under mild conditions to obtain active trimers have failed so far.

Depending on the protein concentration glutamate dehydrogenase tends to associate reversibly to linear associated particles. Cross-linking under these conditions, especially with long chain diimidates, makes the association irreversible. This way one can produce active, non-dissociating polymers similar to those described by Josephs et al. (20).

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