CROSS-LINKING OF SALMONELLA ISOPROPYLMALATE SYNTHASE WITH DIMETHYL SUBERIMIDATE: EVIDENCE FOR ANTAGONISTIC EFFECTS OF LEUCINE AND ACETYL-CoA ON THE QUATERNARY STRUCTURE*

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Summary. Reaction of α-isopropylmalate synthase from Salmonella typhimurium with the bifunctional cross-linking reagent dimethyl suberimidate and subsequent sodium dodecyl sulfate gel electrophoresis results in the appearance of four protein species with molecular weights of about 50,000, 100,000, 150,000, and 200,000. In the absence of ligands, the pattern consists predominantly of tetramers and dimers ("native" pattern). This pattern is changed to one of dimers and monomers in the presence of inhibitory concentrations of leucine. Acetyl-CoA, known to kinetically compete with leucine, restores the "native" pattern. On the basis of previous findings, the results are discussed in terms of "frozen" equilibria.

The regulatory enzyme α-isopropylmalate synthase from S. typhimurium was shown previously by analytical ultracentrifugation to exist in an association-dissociation equilibrium (1,2). It was observed that in the presence of the feedback inhibitor leucine the average molecular weight of the enzyme was shifted towards dissociation (1). While the molecular weight of the denatured monomer was established with two different methods and shown to be about 50,000, it remained uncertain how many monomers constituted the native

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oligomer and what species participated in the equilibrium. Also, indefinite association could not be rigorously ruled out (2). It appeared that some of these questions might be answered by employing cross-linking reagents, particularly in view of the recent work by Davies and Stark (3). These authors, making use of the well-known lysine-linking properties of diimidoesters (4-6), were able to elucidate the subunit make-up of several oligomeric proteins by reaction with dimethyl suberimidate (DMS[1]) followed by SDS gel electrophoresis.

When α-isopropylmalate synthase is subjected to cross-linking with DMS at pH 8.5 and subsequent electrophoresis under denaturing conditions, evidence for a tetrameric structure is obtained. Moreover, pronounced ligand effects can be observed. Thus, incubation with leucine prior to addition of the cross-linking reagent changes the no-ligand pattern of predominantly tetramers and dimers into one of dimers and monomers. Interestingly, the leucine effect can be overcome by acetyl-CoA under conditions similar to those under which competition between the two ligands was shown kinetically (7).

Materials and Methods

1,6 Dicyanohexane ("suberonitrile") was obtained from Eastman, as were acrylamide and N,N'-methylene-bis-acrylamide. SDS was a product of Mann. CoA (Li salt, "chromatopure", 90% reduced CoA) was from P-L Biochemicals, rabbit muscle aldolase from Worthington. DMS dihydrochloride was synthesized from suberonitrile according to the procedure outlined by Davies and Stark (3). It melted at 217-218° (corr.). Analysis: calculated for $C_{10}H_{22}O_2N_2Cl_2$ (273.21): C, 43.96; H, 8.12; N, 10.25; Cl, 25.96. Found: C, 43.93; H, 7.98; N, 10.08; Cl, 26.05. Acetyl-CoA was synthesized from CoA and acetic anhydride, following the procedure of Simon and Shemin (8). All other chemicals were of the best available grade.

[1]Abbreviations: DMS, dimethyl suberimidate; SDS, sodium dodecyl sulfate.

α-Isopropylmalate synthase was isolated from S. typhimurium strain CV-19 essentially as described before (7). In the cross-linking experiments, enzyme was incubated with ligands (replaced with water in control experiments) for 10 min at 23° and pH 8.5 before a freshly prepared solution of neutralized DMS was added. After a time period that varied between 30 min and 3 hrs, an equal volume of a solution containing 2% SDS and 2% mercaptoethanol was added, and the mixture was kept at 37° for 2 hrs. Then, gel electrophoresis in the presence of SDS was performed according to published procedures (1,3,9).

Results and Discussion

The four species of α-isopropylmalate synthase observed after cross-linking with DMS and subsequent SDS gel electrophoresis are multiples of 50,000; the largest oligomer has a molecular weight of about 200,000 (Fig. 1). A tetrameric structure is therefore indicated for the enzyme. Fig. 2 pictures electrophoresis patterns obtained under a variety of conditions. The band

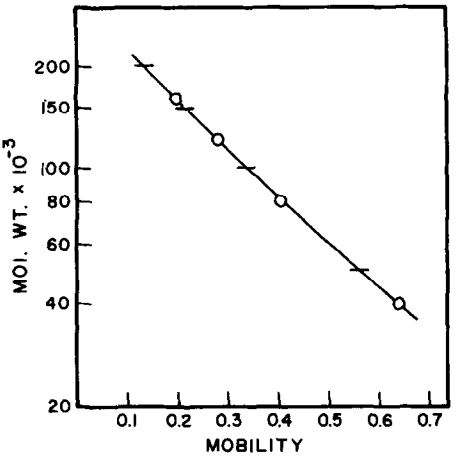

Fig. 1. Molecular weight determination by SDS gel electrophoresis of species obtained after cross-linking with DMS. Open circles: rabbit muscle aldolase, used as standard, assuming molecular weights of 40,000, 80,000, 120,000, and 160,000, respectively (10,11). Horizontal bars: α-isopropylmalate synthase. The concentration of aldolase and synthase during the cross-linking reaction were 4.2 and 0.5 mg/ml, respectively. The concentration of DMS was 2 mg/ml. See "Materials and Methods" for further details. "Mobility" is defined as distance of protein migration/distance of tracking dye migration, corrected for gel stretching.

in gel 1, a control experiment where no cross-linking reagent was used, corresponds to the monomer. Gel 2 is the result of an experiment where native enzyme was exposed to DMS for 3 hrs before being denatured. Bands corresponding to monomers, dimers, trimers, and tetramers are visible, with the tetramers and dimers being most prominent. The tetramer band covers a relatively broad range representing molecular weights between 185,000 and 210,000. It is possible that cross-linking between lysine residues in different locations leads to tetramers of somewhat different shape and rigidity which then behave differently in SDS gel electrophoresis. For gel 3, the enzyme was incubated with kinetically saturating concentrations of acetyl-CoA and α-ketoisocaproate (a sluggishly reacting analog of α-ketoisovalerate, cf. 7). The resulting pattern is similar to the one seen with gel 2, although in some experiments

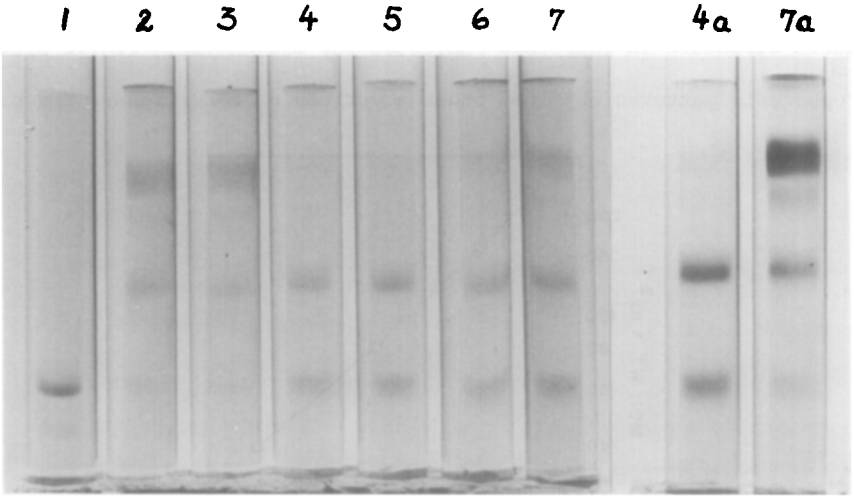

Fig. 2. SDS gel electrophoresis of cross-linked α-isopropylmalate synthase. Treatment of enzyme prior to denaturation: gel No. 1, incubation with 0.2 M triethanolamine-HCl buffer, pH 8.5, for 3 hrs; No. 2, incubation with buffer for 10 min, followed by DMS for 3 hrs; No. 3, incubation with 6.25 mM acetyl-CoA and 6.25 mM α-ketoisocaproate for 10 min, followed by DMS for 3 hrs; Nos. 4 through 7, incubation with 0.25 mM leucine, 6.25 mM α-ketoisocaproate and increasing concentrations of acetyl-CoA for 10 min, followed by DMS for 3 hrs; the acetyl-CoA concentrations were zero (gel No. 4), 0.75 mM (No. 5), 1.5 mM (No. 6), and 6.25 mM (No. 7). Experiments 4a and 7a were identical to 4 and 7, respectively, except that α-ketoisocaproate was omitted. Enzyme concentration: 0.8 mg/ml. DMS concentration: 2 mg/ml (7.4 mM). There was a 1.25 fold dilution upon addition of DMS. All incubations were done at 23°.

the tetramer band appeared to be relatively denser. Gel 4 demonstrates that the presence of leucine causes the tetramers and trimers to disappear in favor of dimers and monomers. This result was obtained with leucine concentrations between 10 mM and 0.25 mM; with 0.1 mM leucine, the tetramers began to reappear. For gels 5 to 7, the leucine concentration during pre-incubation was maintained at 0.25 mM while the concentration of acetyl-CoA was increased as indicated. The α-ketoisocaproate concentration was held constant at 6.25 mM. It is evident that acetyl-CoA reverses the leucine effect, i.e. increasing the acetyl-CoA concentration leads to a more and more distinct return of bands corresponding to tetramers and trimers. Subsequent experiments, documented by gels 4a and 7a, have shown that the ketoacid substrate is not necessary for the effect exerted by acetyl-CoA, implying that acetyl-CoA can bind to the enzyme in the absence of the second substrate. The antagonism between leucine and acetyl-CoA, seen here with respect to the quaternary structure, is strikingly reminiscent of the kinetic competition between the two ligands observed previously under very similar conditions (7).

Other experiments have shown that the results described above are not due to the particular conditions employed. Thus, the electrophoretic patterns seen after 1 hr of reaction with DMS are the same as they are after 3 hrs (at 23°); on the other hand, a reaction time of 30 min is insufficient, as indicated by a relatively strong appearance of monomers. Elevating the DMS concentration to 4 mg/ml or supplying an additional 2 mg/ml in order to compensate for possible loss by hydrolysis does not change the pattern. Increasing the enzyme concentration to 7.2 mg/ml, with DMS being either at 4 or at 8 mg/ml, also does not alter the relative intensity of the bands corresponding to monomers, dimers, trimers, or tetramers.

The question to be asked at this point is how well, if at all, the electrophoretic patterns obtained reflect the true equilibrium situation at the time of

addition of DMS. It is clear that every species smaller than a tetramer could have arisen from incomplete cross-linking. While this is unlikely to happen on the basis of non-availability of lysine residues, since a total of 30 have been determined per 50,000 daltons (12), it could be the result of the gaps between monomers being, or becoming, large enough to make it difficult for DMS to bridge them. Alternatively, the observed patterns could indeed reflect, as a first approximation, "frozen" association-dissociation equilibria. We prefer this latter interpretation on the grounds that several of the findings presented here are in line with what would have been expected from previous molecular weight determinations (1,2,7). In particular, the effect of leucine is very likely dissociative rather than merely conformational (1,2).

In the presence of acetyl-CoA and α-ketoisocaproate, up to 50% of the enzymatic activity of a control is retained even after 3 hrs of reaction with DMS. The residual activity is no longer sensitive to leucine (G. Kohlhaw, to be published). Experiments are now in progress to separate by gel filtration the species obtained after cross-linking in order to find out which of them exhibit activity.

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