JOURNAL OF BACTERIOLOGY, Feb. 1978, p. 802-810
0021-9193/78/0133-0802$02.00/0
Copyright © 1978 American Society for Microbiology
Vol. 133, No. 2
Printed in U.S.A.

Structural Features of Normal and Complemented Forms of the *Neurospora* Isopropylmalate Isomerase

VERNON E. REICHENBECHER, JR., AND S. R. GROSS*

Division of Genetics, Department of Biochemistry, Duke University, Durham, North Carolina 27710

Received for publication 17 August 1977

The isopropylmalate isomerase (EC 4.2.1.33) of *Neurospora crassa* is a globular protein consisting of a single polypeptide chain with a molecular weight of about 90,000. The isomerase cannot easily be freed of a contaminating protease which cleaves the enzyme into two major fragments, one of approximately 56,000 and the other 37,000 daltons. This suggests that the folded polypeptide chain may contain some hinge point or loop exposed on the surface which makes it susceptible to proteolytic attack. Most of the isomerase activity extracted from the wild-type strain is in monomer form. However, a small fraction of the activity in crude extracts is found in multimeric aggregates, and the active isomerase extracted from complementing *leu-2* heterokaryons consists entirely of dimers and higher multimers. These observations suggest that, though active as a monomer, a significant fraction of the normal enzyme might be organized in multimeric form within the cell.

The purification and structural characterization of the isopropylmalate isomerase (the isomerase) of *Neurospora crassa* was undertaken primarily to obtain the elementary information, such as the size and number of subunits of the functional enzyme, necessary for the analysis of the regulatory mechanism governing its synthesis. Two striking features of the genetics of isomerase synthesis and function, however, made a study of the subunit structure of the functional enzyme particularly compelling. First, *leu-3* mutants produce very little isomerase activity (12). Although extensive genetic and biochemical analyses strongly suggested that the *leu-2* gene is the structural gene for the isomerase and that *leu-3* is a gene that specifies a positive regulatory element necessary for isomerase synthesis (20), the gnawing possibility existed that the enzyme might be a heteromultimer containing polypeptides derived from the *leu-3* gene as well as the *leu-2* gene.

The second feature of the functional genetics of the isomerase that required structural analysis of the enzyme is the high frequency and pattern of interallelic complementation displayed by isomerase-deficient mutants. A circular complementation map containing 18 functional segments has been derived from pairwise interactions of *leu-2* mutants in heterokaryons (11). The observation made earlier that the purified functional enzyme is a monomer appeared in conflict with the currently accepted notion that complementation involves subunit interactions of multimeric proteins (5). This forced us to determine the subunit structure of the functional enzyme produced by complementing pairs of *leu-2* mutants. In conformance with current orthodoxy, however, all of the enzymatic activity produced by prototrophic *leu-2* heterokaryons was found associated with multimeric forms of the enzyme.

MATERIALS AND METHODS

Biological. The strains of *N. crassa* used in this study were constructed in this laboratory. Crosses were performed at 25°C on synthetic crossing medium supplemented with the growth factors required by the protoperithecial parent, and the sorbose plating method was used for the isolation of segregants (8). Heterokaryons were formed by the method of Kashmiri and Gross (16). Mycelia were routinely obtained for biochemical analysis after growth in 2-liter Fernbach flasks containing 1 liter of synthetic medium as described by Polacco and Gross (20). When larger amounts of mycelia were required, growth was in 30 liters of medium at 34°C with vigorous aeration. Leucine auxotrophs were grown in medium supplemented with 150 μg of L-leucine per ml.

Preparation of extracts. Ammonium sulfate precipitates were prepared by the method of Gross (12), using 0.1 M potassium phosphate buffer, pH 6.0, containing 0.2 mM L-leucine. A Polytron homogenizer was used instead of a blender to shear the mycelia before sonic treatment. Protamine sulfate (7.5 mg per g [wet weight] of mycelia) was added immediately after sonic disruption rather than after the first centrifugation.

Enzyme assay. Crude ammonium sulfate pellets were dissolved in 0.1 M potassium phosphate buffer, pH 6.0, containing 3 M glycerol, 1 mM dithiothreitol,

and 0.2 mM L-leucine. Although the isomerase has been shown to be inactivated by several sulfhydryl-containing reagents (14), dithiothreitol at this pH and concentration has little, if any, effect on isomerase activity. Assays were performed after removal of debris by centrifugation for 1 h at 100,000 × *g*. The assay of isomerase activity and definition of specific activity is as described by Gross (13). All assays were performed at 34°C in a Gilford spectrophotometer. Protein concentration was determined spectrophotometrically.

Preparation of leucine-Sepharose. Leucine-Sepharose was prepared by the method of Cuatrecasas (6). A 100-ml amount of washed Sepharose 4B was mixed with an equal volume of water, and 30 g of crushed cyanogen bromide was added at once with stirring. The pH was raised to 11 with 8 M NaOH and maintained there during the reaction. The temperature was maintained at 20°C by addition of pieces of ice. After about 0.5 h the reaction was complete (indicated by the cessation of proton release). A large amount of ice was added to the suspension, and the Sepharose was washed with cold 0.1 M sodium borate buffer, pH 9.5. The activated Sepharose was added to a solution of 524 mg (4.0 mmol) of L-leucine in 100 ml of cold 0.1 M sodium borate buffer, pH 9.5. After stirring at 4°C for 18 h, the leucine-Sepharose was collected on a Buchner funnel, washed copiously with water, and stored at 4°C in 0.02% sodium azide.

Polyacrylamide gel electrophoresis. Polyacrylamide gel electrophoresis of native isomerase was performed by the method of Davis (7). Protein bands were stained with Coomassie brilliant blue by the method of Koenig et al. (17). Samples of denatured enzyme for sodium dodecyl sulfate (SDS)-gel electrophoresis were prepared in the following way. Solid guanidine hydrochloride was added to the enzyme solution to a concentration of approximately 6 M (about 50%, wt/vol). The samples were then dialyzed overnight against 0.01 M sodium phosphate buffer, pH 7.2, containing 6 M urea, 1% SDS, and 1% mercaptoethanol, incubated at 37°C for at least 2 h, and heated in a boiling water bath for 1 min. A small amount of bromophenol blue was added as a tracking dye. When necessary, samples were stored at −20°C. SDS electrophoresis of the denatured enzyme was performed and stained with Coomassie brilliant blue according to the procedure of Weber and Osborn (27). Molecular-weight standards were rabbit phosphorylase a (92,000), bovine serum albumin (69,000), catalase (60,000), ovalbumin (43,000), and yeast alcohol dehydrogenase (37,000).

Determination of sedimentation coefficients. The sedimentation coefficient, $s_{20,w}$, of the isomerase was determined by the method of Martin and Ames (18). Glycerol, rather than sucrose, gradients were used, and centrifugation was performed in an SW36 rotor. Hemoglobin served as a standard protein of known $s_{20,w} = 4.3$. Isomerase was assumed to have a partial specific volume of 0.725 cm^3/g.

Determination of Stokes radii. The Stokes radius of the isomerase was calculated from its elution position on a Sephadex G-200 column (43 by 2.5 cm) by the method of Siegel and Monty (22). Values for the parameter *r* were calculated by the method of Ackers (1).

Chemicals. α-Isopropylmalate (α-IPM) and β-IPM were prepared by the method of Calvo and Gross (4). Guanidine hydrochloride (extreme purity) was obtained from Heico, Inc. All other chemicals were obtained from standard commercial sources.

RESULTS

Purification of isopropylmalate isomerase. The method adopted for purification of the isomerase takes advantage of the fact that strains of *Neurospora* which accumulate high concentrations of α-IPM, the inducer of isomerase synthesis, produce large amounts of the enzyme. The double mutant *leu-4*$^{FLR_{92}}$ *leu-1* is such a strain. It overproduces α-IPM due to the presence of a feedback insensitive α-IPM synthase (EC 4.1.3.12) specified by *leu-4*$^{FLR_{92}}$. It also lacks the enzyme β-IPM dehydrogenase (EC 1.1.1.85) and thus accumulates α-IPM due to a block in its utilization. Advantage is also taken of the fact that glycerol and ammonium sulfate stabilize the isomerase (3). High-ionic-strength hydrophobic chromatography, which was used by Bigelis and Umbarger for purification of yeast isomerase (2), also proved extremely useful for purification of the *Neurospora* enzyme.

Purification of the isomerase was accomplished in the following way. Mycelia (113 g, wet weight) of *leu-4*$^{FLR_{92}}$ *leu-1* were washed, disrupted, freed of nucleic acids by precipitation with protamine sulfate (as described above), and fractionated by the addition of solid ammonium sulfate. The protein that precipitated between 45 and 60% saturation contained the vast majority of the isomerase activity. The precipitates could be stored indefinitely at −20°C after collection by centrifugation.

The 45 to 60% ammonium sulfate fraction was dissolved in 65 ml of 0.1 M potassium acetate buffer, pH 6.0. Acetone, cooled to below −20°C, was added slowly with stirring until a 35% (vol/vol) solution was obtained. The temperature was lowered to −15°C, and the precipitate was removed by centrifugation. The acetone concentration of the supernatant solution was adjusted to 50%, and the solution was cooled to −20°C. The precipitate formed was collected by centrifugation.

The 35 to 50% acetone pellet was dissolved in 100 ml of 0.1 M potassium phosphate buffer, pH 6.0, containing 0.2 mM L-leucine and subjected to a second ammonium sulfate fractionation as above. The 45 to 60% saturated ammonium sulfate precipitate II thus obtained was dissolved in a minimal amount of 0.005 M potassium phosphate buffer, pH 6.0, containing 3 M glycerol and desalted on a Sephadex G-25 column equilibrated with the same buffer, followed by chromatography on a diethylamino-

ethyl-cellulose (DE-52) column. The elution pattern is presented in Fig. 1.

The fractions in the shaded region of the activity peak of Fig. 1 were pooled, and enough solid ammonium sulfate was added to bring the solution to 40% saturation (1.6 M). The solution was then applied to a leucine-Sepharose column equilibrated with 0.1 M potassium phosphate buffer, pH 6.0, containing 1.6 M ammonium sulfate and 3 M glycerol and chromatographed with a linearly decreasing ammonium sulfate gradient. The fractions from the front half of the isomerase activity peak were pooled, then frozen and stored at −70°C (Fig. 2). The results of a typical purification are summarized in Table 1.

Polyacrylamide gel electrophoresis. Polyacrylamide disc gel electrophoresis of purified native isomerase from the shaded portion of Fig. 2 yielded a single band containing the vast majority of the applied protein (Fig. 3). Electrophoresis of fractions from the unshaded portions of Fig. 2 yielded an assortment of polypeptides, some of which may have been hydrolytic products of the enzyme (see below). Attempts to elute enzyme activity from these gels after electrophoresis were unsuccessful. This is not surprising, since the enzyme is unstable at the pH at which electrophoresis was performed (pH 8.1 to 8.9).

SDS-disc gel electrophoresis of freshly purified denatured isomerase also showed a single major band (Fig. 3). A plot of the logarithm of the molecular weight versus the electrophoretic mobility in SDS of the isomerase as compared to proteins of known molecular weights yielded an estimated molecular weight for the isomerase of 92,000.

Purified isomerase that had been stored for an extended period of time showed a far different

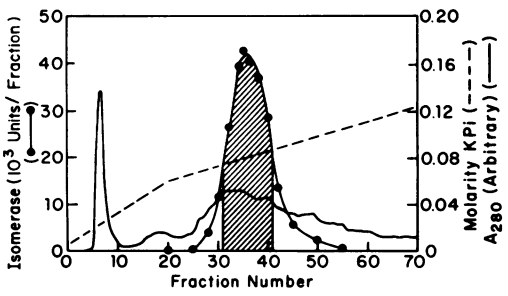

FIG. 1. *DE-52 column chromatography of the isomerase. The 1,000-ml linear gradient of 0.005 to 0.20 M potassium phosphate, pH 6.0, contained 3 M glycerol and was generated by an ISCO Dialagrad pump. The column was 32 by 2 cm, and each fraction contained 10 ml. Chromatography was performed at 4°C.*

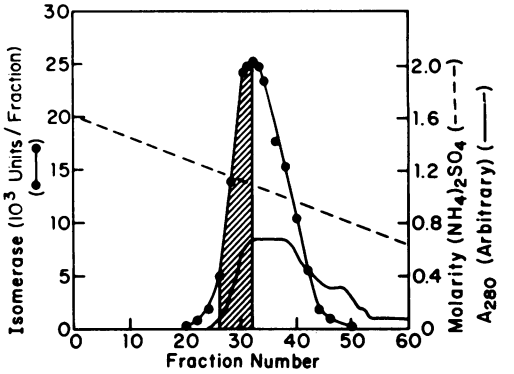

FIG. 2. *Leucine-Sepharose column chromatography of the isomerase. The material chromatographed was from the shaded area of Fig. 1. The 500-ml decreasing linear gradient of 1.6 to 0 M ammonium sulfate in 0.1 M potassium phosphate buffer, pH 6.0, containing 3 M glycerol was generated by an ISCO Dialagrad pump. The column was 20 by 1.1 cm, and each fraction contained 5 ml. Chromatography was performed at 4°C. Fractions from the shaded area were pooled and stored at −70°C.*

SDS-gel electrophoretic pattern. The patterns obtained after various lengths of time at −20°C are presented in Fig. 4. (The samples do not freeze at −20°C due to the large amount of glycerol and ammonium sulfate present.) The intensity of the 92,000-dalton band gradually diminished with time, while new bands of 56,000 and 37,000 daltons increased in intensity. This degradation, which was apparently due to the presence of a protease contaminant, was reduced greatly by storage of the enzyme solution frozen at −70°C. The guanidine hydrochloride treatment routinely used in the preparation of samples for SDS-gel electrophoresis (see Materials and Methods) was introduced in order to rapidly denature the protease(s) and, thus, minimize proteolysis during sample preparation.

Degradation occurred much more rapidly at higher temperatures. The 92,000-dalton band almost completely disappeared after 4 to 8 h of incubation at 34°C. Degradation was inhibited greatly by phenylmethylsulfonyl fluoride and aprotinin, both of which are inhibitors of serine proteases (9, 29). The substrate, α-IPM, at a concentration of 10^{-3} M did not inhibit degradation. Treatment of the isomerase for shorter periods of time with either trypsin or α-chymotrypsin yielded a 56,000-dalton fragment as the major product. It is not clear whether the 37,000-dalton fragment was produced or more rapidly degraded by these proteases, since it did not clearly show up on gels. This fragment, which normally gave a rather weak, diffuse band on SDS-gels (see Fig. 4), may have more sites sus-

TABLE 1. *Isomerase purification*

Step	Volume (ml)	Activity (U)	Protein (mg)	Sp act (U/mg of protein)	Purification factor	Percent recovery
Protamine sulfate supernatant	712	1,310,000	2,770	474	1	100
45–60% saturated $(NH_4)_2SO_4$ precipitate	65	1,170,000	650	1,800	3.80	89.3
35–50% acetone precipitate	100	922,000	—[a]	—	—	70.4
45–60% saturated $(NH_4)_2SO_4$ precipitate II	3.8	517,000	49.5	10,500	22.2	39.5
DE-52 column fractions 31–41	108	320,000	12.3	26,000	54.9	24.4
Leucine-Sepharose column, fractions 26–32	32.2	123,000	2.28	54,000	114	9.4

[a] The protein determination for this sample is unreliable due to the presence of much insoluble material.

ceptible to protease action than the larger fragment and, thus, be more sensitive to further degradation.

Amino acid analysis. The amino acid composition of the isomerase, determined after acid hydrolysis of the purified lyophilized protein, was rather undistinguished (Table 2). The amino terminus of the intact enzyme appeared to be blocked, since no end group was detected by the dansylation method (10, 28). Serine was detected as the amino-terminal residue of the purified large fragment (generated by the contaminating protease), while the small fragment appeared heterogeneous, yielding phenylalanine, threonine, serine, and smaller amounts of glycine and valine. The observed *N*-terminal heterogeneity of the smaller fragment is not surprising because it almost always appeared as a diffuse band on SDS-gels, suggestive of size heterogeneity. Since the dansylation method yielded amino termini for both fragments while none was detected for the intact polypeptide, the blocked amino acid or peptide containing it must have been cleaved off during hydrolysis. Because of this, it is impossible to orient the molecule with respect to the two fragments on the basis of end group analysis.

Molecular weight of purified isomerase. The sedimentation coefficient of purified isomerase was determined to be 5.6S by sedimentation velocity in a glycerol density gradient (Fig. 5). Hemoglobin ($s_{20,w}$ = 4.3) served as a marker protein.

The Stokes radius of purified isomerase was determined on the basis of its elution profile from Sephadex G-200 by the method of Siegel and Monty (22). The column was equilibrated with 0.1 M potassium phosphate buffer, pH 6.0, containing 3 M glycerol. Bacteriophage f1 (provided by John Woolford) served as a marker for the column void volume, while D-[U^{14}C]lactic acid was a marker for the total internal volume of the column accessible to solvent. Bovine serum albumin served as a marker protein of Stokes radius = 3.5 nm. By this method, purified isomerase was determined to have a Stokes radius of 3.83 nm (Fig. 6).

Calculations based on equations 1 and 2 of Siegel and Monty (22), using the experimentally determined values for Stokes radius, $s_{20,w}$, and an assumed partial specific volume consistent with the amino acid composition of 0.725 cm^3/g, yielded a value of 88,500 as the molecular weight of purified native isomerase. The frictional ratio, f/f_{min}, of purified isomerase was calculated to be 1.30. Proteins with frictional ratios in this range are considered to be globular.

The calculated molecular weight of 88,500 agrees well with the value 80,000 to 90,000 reported previously using cruder isomerase preparations (15). Equilibrium centrifugation data on purified native isomerase could not be obtained because the preparation was subject to proteolysis by the protease contaminant under the conditions of the centrifugation. However, equilibrium centrifugation of purified isomerase in 6 M guanidine hydrochloride indicated a molecular weight of approximately 87,000. Since the molecular weight of the native isomerase corresponds with the molecular weight of the reduced, denatured isomerase as estimated from SDS-gels, the purified native isomerase must be monomeric in structure.

Enzyme derived from complementation interactions. The structure of the isomerase is specified by the *leu-2* gene, and interallelic complementation interactions between *leu-2* mutants are common. The complementation map derived for *leu-2* is circular (11). An enzyme capable of activation by interallelic complementation interactions, however, would be expected to be a multimeric protein, consisting of two or more identical subunits (5). The data presented above, however, indicate that the isomerase is a monomer. Therefore, the properties of the isomerase derived from complementing *leu-2*

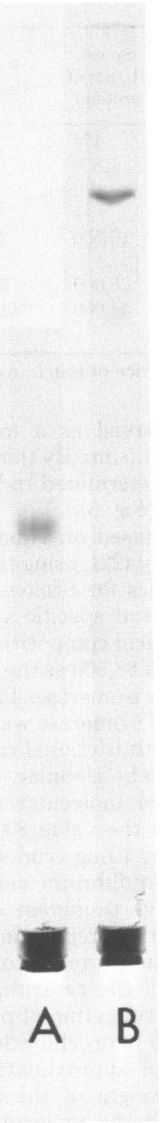

FIG. 3. *Polyacrylamide gel electrophoresis of purified isomerase. (A) Electrophoresis of the native enzyme in a 7.5% acrylamide gel at 2 mA per tube for 3 h at 4°C. The upper buffer chamber contained 0.05 M tris(hydroxymethyl)aminomethane-glycine, pH 8.9, and the lower buffer chamber contained 0.1 M tris(hydroxymethyl)aminomethane-hydrochloride, pH 8.1. (B) SDS-gel electrophoresis of denatured purified isomerase. The sample was prepared as described in Materials and Methods. Electrophoresis was performed at 3 mA per tube for 19 h at 25°C. The 7.5% acrylamide gel contained 0.1 M sodium phosphate buffer, pH 7, 0.02 M disodium ethylenediaminetetraacetic acid, 0.1% SDS, and 6 M urea. The electrophoresis buffer contained 0.1 M sodium phosphate buffer, pH 7, 0.02 M disodium ethylenediaminetetraacetic acid, and 0.1% SDS.*

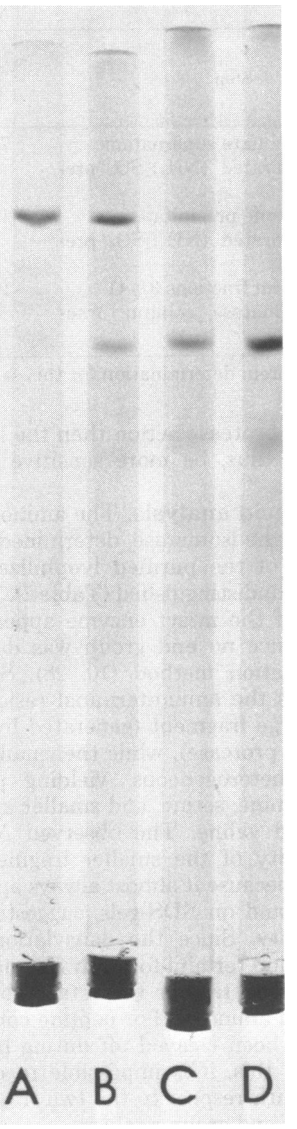

FIG. 4. *Degradation of the isomerase during storage. SDS-gel electrophoresis of samples of purified isomerase stored as follows: (A) fresh preparation; (B, C, D) after 8, 20, and 59 days of storage at −20°C. Details of the electrophoresis were as described in Fig. 3.*

heterokaryons were compared with those of the wild-type enzyme in order to reconcile the difference between the predicted and observed structure.

Six *leu-2* mutants which complement efficiently were chosen for analysis from positions on the complementation-map such that nine of the possible pairwise combinations formed prototrophic heterokaryons. The mutants and their

TABLE 2. *Amino acid analysis of purified isomerase*[a]

Amino acid	Mole fraction	Residues/90,000-dalton chain
Lys	0.0660	55.1
His	0.0218	18.2
Arg	0.0457	38.1
Asx	0.102	85.1
Thr	0.0649	54.1
Ser	0.0715	59.6
Glx	0.115	95.9
Pro	0.0452	37.7
Gly	0.0935	78.0
Ala	0.0946	78.9
Half-Cys	0.0147	12.3
Val	0.0627	52.3
Met	0.0201	16.8
Ile	0.0530	44.2
Leu	0.0687	57.3
Tyr	0.0207	17.3
Phe	0.0346	28.9
Trp	0.0058	4.8

[a] Values for serine and threonine were extrapolated to zero time. Valine and isoleucine were determined after 96 h of hydrolysis. Tryptophan was determined after hydrolysis by methanesulfonic acid (47). Half-cystine was determined as cysteic acid after performic acid oxidation (28).

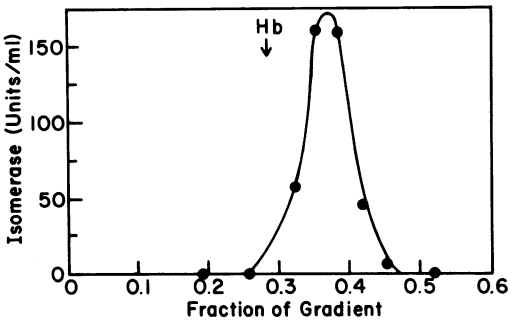

FIG. 5. *Sedimentation velocity of the purified isomerase in a glycerol gradient. A linear 10 to 30% glycerol gradient in 0.1 M potassium phosphate buffer, pH 6.0, was used. Bovine hemoglobin (Hb) served as a marker protein and was determined by its absorbance at 410 nm. Centrifugation was at 36,000 rpm in an SW36 rotor for 36 h at 4°C. The top of the gradient is at the left.*

complementation groups (see reference 11 for nomenclature) were: D189 (D28 group), D83 (D72 group), R86 (D19 group), D43 (D43 group), D161 (D42 group), and DR1 (complementation group unknown, but from the same region of the map as D43 and D161). None of the six *leu-2* mutants as homokaryons produced more than a trace of isomerase activity. Each of the *leu-2* mutants was crossed with *leu-4*$^{FLR_{92}}$ in order to insert the feedback-negative α-IPM synthase into both sides of the heterokaryon to increase

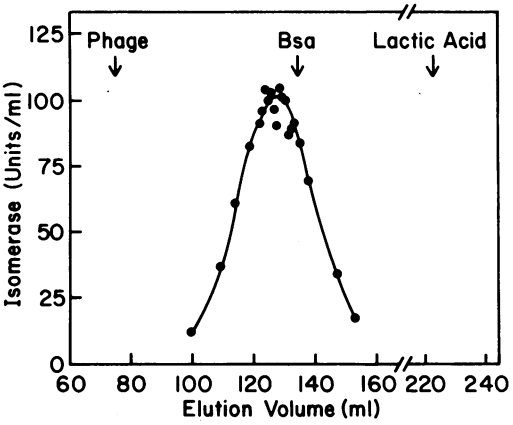

FIG. 6. *Sephadex G-200 chromatography of purified isomerase. The arrows show the positions of the elution peaks of f1 bacteriophage, bovine serum albumin (Bsa), and D-[U-*^{14}*C]lactic acid.*

the endogenous production levels of the mutant defective isomerase by relieving feedback inhibition of α-IPM synthesis.

Properties of the isomerase from complementing *leu-2* heterokaryons. The first indication of a difference between the isomerase produced by the heterokaryons and that of the wild-type strain was that most of the isomerase activity from heterokaryons precipitated at a lower ammonium sulfate concentration (30 to 45% saturated) than the normal enzyme (45 to 60% saturated). The sedimentation coefficients and Stokes radii of the isomerase in crude ammonium sulfate fractions (30 to 45% saturated) prepared from three of these heterokaryons were determined. Each of the three presumed hybrid enzymes yielded a single peak of activity upon glycerol density gradient centrifugation of $s_{20,w}$ = 6.0 to 7.1 (Table 3). Sephadex G-200 chromatography of extracts of two of the heterokaryons, D43/D189 and DR1/D83, yielded single peaks of activity at a position indicating a Stokes radius of about 6.8 nm (Fig. 7A and B). The third extract, D161/R86, yielded a shoulder of activity (peak II) in the 6.8-nm region, but the bulk of the activity was found in a region (peak I) just behind the void volume of the column (Fig. 7C). The Stokes radius of these aggregates was calculated to be in the vicinity of 11.1 nm, but the error is probably great because of lack of resolution from the front. Activity associated with aggregates of this size was not found after glycerol density gradient centrifugation. Failure to find the large aggregates after centrifugation may be indicative of either size heterogeneity, in which case the small amount of activity might have been distributed widely in the gradient, or dissociation of the large aggregates during den-

TABLE 3. *Properties of wild-type isomerase and isomerase from complementing leu-2 heterokaryons*[a]

Source	$s_{20,w}$	Stokes radius (nm)	Mol wt	f/f_{min}
Purified wild-type isomerase	5.6	3.83	88,500	1.30
D43/D189	6.0	6.83	169,000	1.87
DR1/D83	7.1	6.78	199,000	1.76
D161/R86, peak II	6.5	6.88	185,000	1.83
D161/R86, peak I		11.1[b]		

[a] All *leu-2* mutants used in forming heterokaryons contained the FLR92 allele of *leu-4*. The data shown for heterokaryons was obtained using crude 30 to 45% $(NH_4)_2SO_4$ extracts.

[b] Rough estimate. The potential for error in this number is great, since the enzyme peak eluted very close to the void volume of the Sephadex G-200 column.

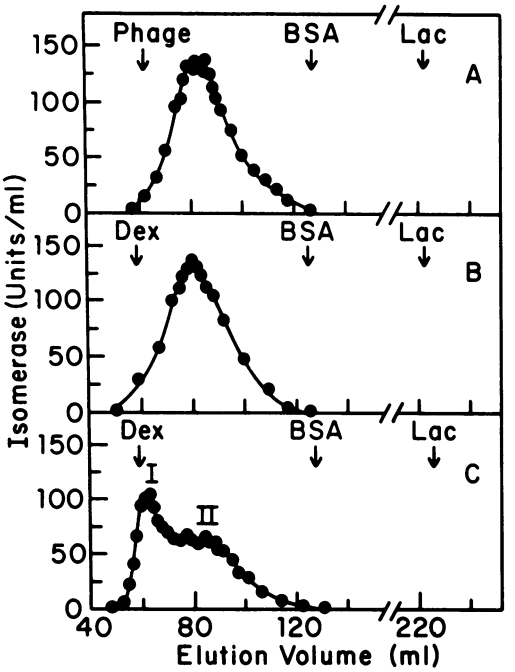

FIG. 7. *Sephadex G-200 chromatography of isomerase from complementing leu-2 heterokaryons. The arrows show the positions of the elution peaks of f1 bacteriophage, blue dextran 2000 (Dex), bovine serum albumin (BSA), and D-[U-^{14}C]lactic acid (Lac). The 30 to 45% saturated $(NH_4)_2SO_4$ extracts used were obtained from the heterokaryons (A) D43/D189, (B) DR1/D83, and (C) D161/R86.*

sity gradient centrifugation.

The sedimentation coefficients and Stokes radii determined for the isomerase from each of the three heterkaryons examined, along with calculated molecular weights and frictional ratios, are summarized in Table 3. Each heterokaryon contained an isomerase with a molecular weight about twice that of purified wild-type isomerase. The frictional ratio of the "dimer" isomerase from these heterokaryons was calculated to be about 1.8. This is significantly higher than that usually obtained for globular proteins (25, 26) and, assuming a normal degree of hydration, indicates that the dimer isomerase has a highly elongated form. The monomeric form of the isomerase had a frictional ratio of about 1.3, which is in the normal range for globular proteins. The large increase in the frictional ratio yielded a low sedimentation coefficient for the dimer which by coincidence was only slightly larger than that of the monomer isomerase. This coincidence misled Gross and Webster (15) to the conclusion that the isomerase from complementing *leu-2* heterokaryons was the same size as the normal enzyme.

Sephadex G-200 chromatography of crude wild-type isomerase. Only the isomerase monomer was observed in purified preparations of the wild-type enzyme. However, since the bulk of the normal enzyme was found to precipitate in the 45 to 60% saturated ammonium sulfate fraction, it seemed likely that dimers and larger aggregates may have been discarded during purification. To determine whether dimers and higher multimers were produced in *leu-2*$^+$ strains, a 30 to 45% saturated ammonium sulfate precipitate was prepared from the *leu-4*$^{FLR_{92}}$ *leu-1* double mutant, which produces the normal isomerase in excess. The elution profile of isomerase activity in the extract from a Sephadex G-200 column is shown in Fig. 8. The majority of activity eluted in the

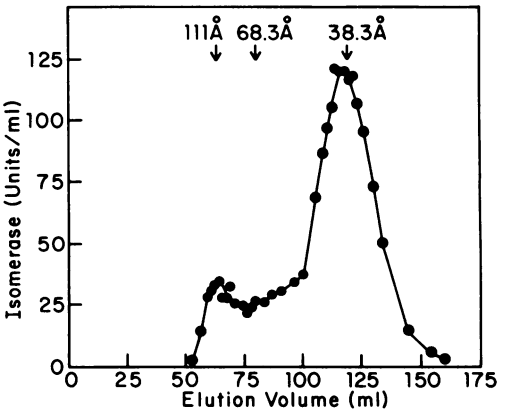

FIG. 8. *Sephadex G-200 chromatography of a crude extract of wild-type isomerase. The 30 to 45% saturated $(NH_4)_2SO_4$ extract used was obtained from a leu-4*$^{FLR_{92}}$ *leu-1 double mutant. The arrows mark the position expected for elution of molecules of the indicated Stokes radii.*

monomer region (approximate Stokes radius of 3.8 nm), but some activity was found in the dimer region (approximately 6.8 nm), and a peak can be seen in the region of larger aggregates (approximately 11.1 nm). Thus, at least a small fraction of the normal enzyme can be found in multimeric form.

DISCUSSION

The results presented here indicate that purified wild-type *Neurospora* isomerase is a globular protein consisting of a single polypeptide chain with a molecular weight of about 90,000. It seems likely that the folded polypeptide chain contains some sort of hinge point or loop exposed on the surface of the protein, making it susceptible to site-specific endolytic cleavage. The purified isomerase preparation used here was contaminated by a small amount of protease, which cleaves the native enzyme into two major fragments: one of 56,000 daltons and one less uniform in size but averaging 37,000 daltons. This cleavage pattern is at least partially reproduced by both trypsin and chymotrypsin. The 37,000-dalton proteolytic fragment may be especially susceptible to further degradation, as evidenced by its amino-terminal end group and apparent size heterogeneity. The identity of the protease naturally contaminating the purified isomerase preparation is unknown, but its mode of action and the fact that it is inhibited by serine protease inhibitors are reminiscent of the alkaline endopeptidase of *Neurospora* studied by Siepen et al. (23, 24).

Samples of isomerase in which approximately 75% of the enzyme molecules have been cleaved have been found to retain a large fraction of the original isomerase activity, and samples in which all the molecules have been cleaved retained about 30% of the original activity (unpublished observations). Hence, either one of the proteolytic fragments is enzymatically active by itself or the two fragments can still associate to form an active enzyme molecule even after the polypeptide backbone has been clipped.

Extensive genetic and physiological investigations have shown that the product of the *leu-3* cistron is a positive regulatory element involved in controlling the rate of synthesis of the isomerase, the dehydrogenase, and the enzymes of the isoleucine-valine biosynthetic pathway (19, 20). Earlier speculation that *leu-3* as well as *leu-2* contributed one or more polypeptide subunits to the enzyme was based on the fact that *leu-3* mutants lack isomerase activity and that the presence of a *leu-3* allele inhibits interallelic complementation interactions at the *leu-2* locus (11). The demonstration here that the isomerase consists of only one kind of polypeptide chain provides further evidence against the possibility that *leu-3* contributes an additional subunit necessary for enzyme activity. Similarly, since the yeast isomerase was found to consist of a single polypeptide chain (3), it seems likely that five of the six genes involved in isomerase synthesis in yeast (21) have a regulatory rather than a structural role.

One of the perplexing problems of analyses of the functional architecture of the cell is that most enzymes must be extracted from their natural surroundings and purified before study. Extraction and purification might be expected to dissociate weakly associated aggregates and, as a consequence, obscure subtle intermolecular interactions of physiological importance. In the absence of information about interallelic complementation, the isolation of the isomerase as a single functional polypeptide would not have raised questions about the structural organization of the enzyme in vivo. However, the fact that complementation among *leu-2* alleles is common and often efficient (11) suggested the likelihood that at least a fraction of the isomerase in vivo exists as multimers of two or more identical polypeptides (5). This notion was confirmed by the observation that the active isomerase extracted from complementing *leu-2* heterokaryons consisted entirely of dimers and larger aggregates (higher multimers or mixed complexes). Indeed, a closer examination of crude enzyme preparations obtained from a strain producing the normal enzyme revealed that a small fraction of the activity can be found as dimers and aggregates of higher multimeric composition.

The question then arises of whether the enzyme functions normally within the cell in multimeric form and is dissociated upon extraction or whether, instead, the ratio of monomeric to multimeric forms found in extracts is a reasonable approximation of the state of aggregation of the enzyme within the cell. Several observations suggest that dissociation is favored in vitro. First, monomer-to-multimer transitions have not been observed at any stage of purification or analysis of the isomerase. Second, disaggregation seems likely to account for failure to find aggregates larger than dimers after glycerol density centrifugation. Finally, countless attempts have been made, without success, to obtain in vitro complementation using extracts from strains that complement efficiently in vivo. While these kinds of negative data are insufficient to resolve this problem, the complementation interaction is sufficient in itself to suggest that a functionally significant portion of the

isomerase is aggregated in vivo.

The consistence of the complementation interaction and the complexity of the circular complementation map derived for the isomerase (11) suggests the involvement of highly specific structural interactions between aggregating monomers. In fact, the high frictional ratio of the dimers, which suggests that they have a much more elongated structure than would be expected from the association of two globular subunits, is indicative of extensive conformational changes upon aggregation. Thus the multimers may have many more specific interacting contact points than might have been expected from interactions between globular subunits.

ACKNOWLEDGMENTS

We wish to thank Jacqueline Reynolds for performing the sedimentation equilibrium determination and for many helpful discussions and H. Steinman and J. Abernethy for the end group and amino acid determinations.

V.E.R. was a predoctoral trainee supported by a Public Health Service training grant (GM 02007) from the National Institute of General Medical Sciences. This work was supported by a grant from the National Science Foundation (PCM 74-03018).

LITERATURE CITED

1. **Ackers, G. K.** 1964. Molecular exclusion and restricted diffusion processes in molecular-sieve chromatography. Biochemistry **3**:723–730.
2. **Bigelis, R., and H. E. Umbarger.** 1975. Purification of yeast α-isopropylmalate isomerase. High ionic strength hydrophobic chromatography. J. Biol. Chem. **250**:4315–4321.
3. **Bigelis, R., and H. E. Umbarger.** 1976. Yeast α-isopropylmalate isomerase. Factors affecting stability and enzyme activity. J. Biol. Chem. **251**:3545–3552.
4. **Calvo, J. M., and S. R. Gross.** 1970. Isolation and chemical estimation of α-isopropylmalate and β-isopropylmalate, p. 791–793. *In* H. Tabor and C. W. Tabor (ed.), Methods in enzymology, vol. 17A. Academic Press Inc., New York.
5. **Crick, F. H. C., and L. E. Orgel.** 1964. The theory of inter-allelic complementation. J. Mol. Biol. **8**:161–165.
6. **Cuatrecasas, P.** 1970. Protein purification by affinity chromatography. J. Biol. Chem. **245**:3059–3065.
7. **Davis, B. J.** 1964. Disc electrophoresis. II. Method and application to human serum proteins. Ann. N.Y. Acad. Sci. **121**:404–427.
8. **Davis, R. H., and F. J. deSerres.** 1970. Genetic and microbiological techniques for *Neurospora crassa*, p. 79–143. *In* H. Tabor and C. W. Tabor (ed.), Methods in enzymology, vol. 17A. Academic Press Inc., New York.
9. **Fahrney, D. E., and A. M. Gold.** 1963. Sulfonyl fluorides as inhibitors of esterases. I. Rates of reaction with acetylcholinesterase, α-chymotrypsin, and trypsin. J. Am. Chem. Soc. **85**:997–1000.
10. **Gray, W. R.** 1972. End-group analysis using dansyl chloride, p. 121–138. *In* C. H. W. Hirs and S. N. Timasheff (ed.), Methods in enzymology, vol. 25. Academic Press Inc., New York.
11. **Gross, S. R.** 1962. On the mechanism of complementation at the *leu-2* locus of *Neurospora*. Proc. Natl. Acad. Sci. U.S.A. **48**:922–930.
12. **Gross, S. R.** 1965. The regulation of synthesis of leucine biosynthetic enzymes in *Neurospora*. Proc. Natl. Acad. Sci. U.S.A. **54**:1538–1546.
13. **Gross, S. R.** 1970. Isopropylmalate isomerase (*Neurospora*), p. 786–790. *In* H. Tabor and C. W. Tabor (ed.), Methods in enzymology, vol. 17A. Academic Press Inc., New York.
14. **Gross, S. R., R. O. Burns, and H. E. Umbarger.** 1963. The biosynthesis of β-carboxy-β-hydroxyisocaproate and α-hydroxy-β-carboxyisocaproate. Biochemistry **2**:1046–1052.
15. **Gross, S. R., and R. E. Webster.** 1963. Some aspects of interallelic complementation involving leucine biosynthetic enzymes of *Neurospora*. Cold Spring Harbor Symp. Quant. Biol. **28**:543–547.
16. **Kashmiri, S. V. S., and S. R. Gross.** 1970. Mutations affecting the regulation of the enzymes of leucine synthesis in *Neurospora*. Genetics **64**:423–440.
17. **Koenig, R., H. Stegemann, H. Francksen, and H. L. Paul.** 1970. Protein subunits in the potato virus X group. Determination of the molecular weights by polyacrylamide electrophoresis. Biochim. Biophys. Acta **207**:184–189.
18. **Martin, R. G., and B. N. Ames.** 1961. A method for determining the sedimentation behavior of enzymes: application to protein mixtures. J. Biol. Chem. **236**:1372–1379.
19. **Olshan, A. R., and S. R. Gross.** 1974. Role of the *leu-3* cistron in the regulation of the synthesis of isoleucine and valine biosynthetic enzymes of *Neurospora*. J. Bacteriol. **118**:374–384.
20. **Polacco, J. C., and S. R. Gross.** 1973. The product of the *leu-3* cistron as a regulatory element for the production of the leucine biosynthetic enzymes of *Neurospora*. Genetics **74**:443–459.
21. **Satyanarayana, T., H. E. Umbarger, and G. Lindegren.** 1968. Biosynthesis of branched-chain amino acids in yeast: correlation of biochemical blocks and genetic lesions in leucine auxotrophs. J. Bacteriol. **96**:2012–2017.
22. **Siegel, L. M., and K. J. Monty.** 1966. Determination of molecular weights and frictional ratios of proteins in impure systems by use of gel filtration and density gradient centrifugation. Application to crude preparations of sulfite and hydroxylamine reductase. Biochim. Biophys. Acta **112**:346–362.
23. **Siepen, D., and M.-R. Kula.** 1976. Specificity of five intracellular proteinases of *Neurospora crassa*. FEBS Lett. **66**:31–34.
24. **Siepen, D., P.-H. Yu, and M.-R. Kula.** 1975. Proteolytic enzymes of *Neurospora crassa*. Purification and some properties of five intracellular proteinases. Eur. J. Biochem. **56**:271–281.
25. **Smith, M. H.** 1970. Molecular weights of proteins and some other materials including sedimentation, diffusion, and frictional coefficients and partial specific volumes, p. C-3. *In* H. A. Sober (ed.), CRC handbook of biochemistry, selected data for molecular biology, 2nd ed. The Chemical Rubber Co., Cleveland.
26. **Tanford, C.** 1961. Physical chemistry of macromolecules. John Wiley and Sons, Inc., New York.
27. **Weber, K., and M. Osborn.** 1969. The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. J. Biol. Chem. **244**:4406–4412.
28. **Weiner, A. M., T. Platt, and K. Weber.** 1972. Amino-terminal sequence analysis of proteins purified on a nanomole scale by gel electrophoresis. J. Biol. Chem. **247**:3242–3251.
29. **Werle, E.** 1972. Trasylol: a short survey on its history, biochemistry, and activities, p. 9–16. *In* W. Brendel and G. L. Haberland (ed.), New aspects of trasylol therapy, vol. 5. Protease inhibition in shock therapy. Schattauer, Stuttgart, New York.