The α-Isopropylmalate Synthetase of *Neurospora*. II. The Relation between Structure and Complementation Interactions*

R. E. Webster,† Charles A. Nelson, and S. R. Gross

ABSTRACT: α-Isopropylmalate synthetase has a molecular weight of about 143,000 and consists of three identical subunits. Synthetases obtained from complementation interactions are about the same size and shape as the normal enzyme but are composed of differently defective subunits. Each of the hybrid enzymes obtained differs from each other and the normal enzyme with respect to sensitivity to thermal inactivation, catalysis, and several properties associated with allosteric interactions between substrate and the end product inhibitor, leucine. The general increase in thermal sensitivity and the changes observed in the allosteric response suggest the involvement of local conformational irregularities in the hybrid enzymes.

The results of the investigation reported in the preceding paper (Webster and Gross, 1965) indicate strongly that α-isopropylmalate synthetase is an allosteric protein subject to end product inhibition. The enzyme, however, is of interest from yet another point of view, for active synthetase can be formed as a consequence of complementation interactions between defective products of the *leu*-4 cistron of *Neurospora* (Gross and Webster, 1963). Furthermore, the interactions between pairs of allele products are systematically related to each other for they can be represented in a linear overlapping array, or complementation map, by using the simple assumption that gene products that complement do not share overlapping defects (Catcheside and Overton, 1958).

Most investigations of the phenomenon of interallelic complementation have been related only to genetic and functional aspects of the interaction, for rarely has the protein involved been available for study. However, in at least two instances, glutamic dehydrogenase (Fincham and Coddington, 1963) and alkaline phosphatase (Schlesinger and Levinthal, 1963), the pure enzyme has been available for chemical and physical analysis. In both cases the protein has been found to be a multimer consisting of several identical subunits. Convincing evidence has been obtained, especially for alkaline phosphatase, that complementation involves the polymerization of differently defective subunits yielding a catalytically active heteropolymer (Crick and Orgel, 1964).

The availability of relatively pure synthetase not only affords the possibility of studying the mechanism of complementation but more interestingly the complementation interaction itself provides a very large number of differently substituted active proteins for the study of the relation between protein structure and catalytic activity. In this paper the synthetase is shown to be a multimer most probably consisting of three identical subunits. In addition, the kinetic, allosteric, and substrate-binding properties of several hybrid enzymes (for ease of reference, enzymes obtained from complementation interactions are referred to as hybrid enzymes) are described in relation to the efficiency of the complementation interactions involved and the conformational properties of the enzymes produced.

Experimental Methods and Procedures

Biological. All *leu*-4 mutations studied were induced with ultraviolet irradiation and obtained by the inositol-less death selection procedure (Lester and Gross, 1959; Gross and Gross, 1961).

Maximum derepression of synthetase synthesis was required in order to ensure sufficient yield of hybrid enzyme. In order to accomplish this, triple mutants were synthesized with the following constitution: *leu*-4, *leu*-1 (D221), and either *inos* (inositol-requiring 89601) or *pan* (pantothenic acid requiring 5531). Derepression of synthetase production could be obtained by growth of a heterokaryon consisting of two such *leu*-4 triple mutants (S. R. Gross, in preparation) on limiting leucine in the absence of pantothenic acid and inositol. (Neither pantothenic acid nor inositol was supplied in order to ensure heterokaryotic growth.) In general *leu*-4 mutants that mapped on the left side of the complementation map (Figure 1) were put in combination with *leu*-1 and *inos*, and those on the right with *leu*-1 and *pan*.

Hybrid synthetase was obtained from heterokaryotic mycelia grown for 48 hr at 34° with aeration in 6 l. of

* From the Departments of Microbiology and Biochemistry, Duke University, Durham, N. C. *Received June 1, 1965.* Supported by a research grant (GM 07250) from the National Institutes of Health, U. S. Public Health Service, and by a grant (GB-727) from the National Science Foundation.

† Present address: Rockefeller Institute, New York, N. Y.

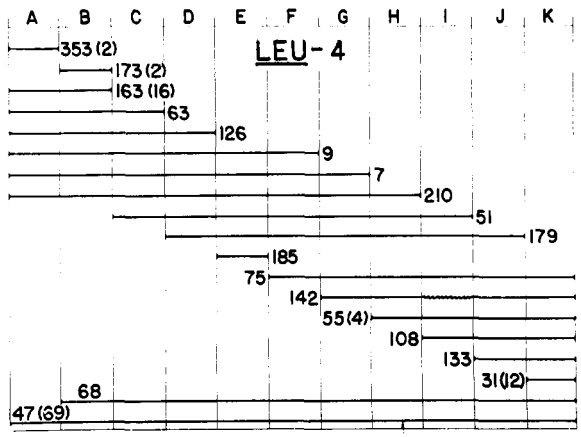

FIGURE 1: The complementation map of the *leu*-4 gene of *Neurospora crassa*. Groups of mutants demonstrating similar patterns are referred to by that member of the group with the lowest numerical designation, and the number of mutants comprising the group is noted in parentheses. Letter prefixes denoting laboratory of origin of the mutants are not included in the figure. This figure is reproduced from an article by Gross and Webster [(1963), *Cold Spring Harbor Symp. Quant. Biol. 28*, 543] with the permission of the director.

synthetic medium with 15 mg of DL-leucine/l. and 1% sucrose. Growth was initiated with 10 ml of a dense conidial suspension (OD_{550} 0.5) obtained from heterokaryons grown previously from mixed conidial inocula on solid minimal medium supplemented with 150 mg/l. of DL-leucine.

Most of the experiments reported here were performed on preparations of hybrid α-isopropylmalate synthetase purified through the second ammonium sulfate precipitation step described in the preceding paper (Webster and Gross, 1965) with the exception that in the last step 11 ml of saturated ammonium sulfate was added instead of 20 ml/100 ml of the supernatant solution. The ammonium sulfate precipitates contained the synthetase virtually free of contaminating deacylase. Whenever necessary, for comparative purposes, normal synthetase was prepared in the same way either from the wild-type strain STD4A or from the high synthetase yielding *leu*-1 mutant strain D6:23A.

The highly purified normal α-isopropylmalate synthetase preparations used in the physical and chemical studies were obtained by the purification procedure described in the preceding paper (Webster and Gross, 1965).

Chemical. Amino acid analysis and tryptic digestion were performed on enzyme protein, freed of leucine by passage through a G-50 Sephadex column (20 × 1.3 cm) equilibrated with 0.1 M potassium phosphate buffer, pH 6.5. The protein was then denatured by heat and the precipitate was washed several times with water to remove salt and any residual trace of leucine. The amino acid composition of the synthetase was determined by chromatographic analysis after 24-hr acid hydrolysis of the protein according to the procedure of Spackman *et al.* (1958). Tryptophan content was estimated by the spectrophotometric method of Bencze and Schmid (1957) and the colorimetric procedure of Spies and Chambers (1948, 1949). Half-cystine was determined as cysteic acid after performic acid oxidation (Moore, 1963). Sulfhydryl groups were estimated in 0.1 M phosphate buffer, pH 7.0, with *p*-mercuribenzoate (PCMB)[1] according to the method of Boyer (1954).

Trypsin digestion of the synthetase was carried out in the following manner: 0.3 mg of trypsin in 0.001 M HCl was added to 10 mg of the washed heat-precipitated protein suspended in 1.5 ml of distilled water, and the pH was adjusted to 8.0. After a 4-hr incubation at 40°, an additional 0.1 mg of trypsin was added and the reaction was allowed to continue for an additional hour. The incubation mixture was adjusted to pH 6.5 and the very small amount of precipitate that appeared was removed by centrifugation.

Peptide patterns were resolved by electrophoresis and chromatography by the method described for fumarase by Kanarek *et al.* (1964). Electrophoresis of tryptic digests were carried out in 0.2 M pyridine–sodium acetate buffer, pH 6.5, for 180 min with a gradient of 15 v/cm. Chromatography was carried out with a pyridine–1-butanol–acetic acid–water mixture (100:150:30:120 by volume) for 12.5 hr. Peptide patterns were visualized with ninhydrin (Levy and Chung, 1953); histidine-containing peptides were detected by spraying with diazotized sulfanilic acid (Block, 1951).

For most chemical and physical studies reported here, the amount of protein was determined directly by weighing heat-denatured washed and freshly lyophilized protein. When solutions of the purified protein were necessary, as was the case for PCMB titration of free thiol groups, protein concentration was determined spectrophotometrically at 280 mμ in 0.1 M phosphate buffer, pH 7.0, using an empirically derived $E^{1\%}_{1cm}$ of 11.9. The extinction coefficient could not be determined accurately by weight due to difficulties encountered in obtaining substantial amounts of soluble salt-free protein. Instead the extinction coefficient was calculated from a comparison of the absorption at 280 mμ of a purified synthetase solution with the value obtained on the same sample by Waddell's 215–225 mμ absorption method (Murphy and Kies, 1960) and the estimate of protein concentration by the trichloroacetic acid precipitation method of Bücher (1947). For purposes other than structural analysis, protein was determined by the methods of Warburg and Christian (1941) and Lowry *et al.* (1951).

Guanidine hydrochloride was prepared from guanidine carbonate by the method of Anson (1941).

Physical. Sedimentation velocity measurements of the synthetase were made in 0.1 M phosphate buffer, pH 6.5, containing 2×10^{-4} M L-leucine (phosphate–leucine buffer) with 12-mm single-sector cells in a Beckman/

[1] Abbreviations used throughout the text: PCMB, *p*-mercuribenzoate; α-KIV, α-ketoisovalerate; CoA, coenzyme A; NEM, *N*-ethylmaleimide.

Spinco Model E ultracentrifuge at 59,780 rpm at 25°. Measurements in 6 M guanidine hydrochloride, 0.1 M β-mercaptoethanol, and 0.01 M Tris–HCl buffer, pH 8.0, were performed using a synthetic-boundary cell at 42,040 rpm.

Sedimentation equilibrium studies were carried out at relatively high speeds with short protein columns as described by Yphantis (1964). The initial protein concentrations were about 0.2 mg/ml and the sedimentations were carried out at 25°.

The partial specific volume of the protein was calculated to be 0.73 ml/g in buffer from the amino acid composition according to the method of McMeekin *et al.* (1949) and 0.71 ml/g in guanidine hydrochloride. The partial specific volume of most proteins decreases about 0.02 ml/g upon denaturation in guanidine hydrochloride (Kielley and Harrington, 1960; Marler and Tanford, 1964). It should be noted that a difference in partial specific volume of 0.005 would alter the molecular weight in guanidine by 2%. The densities for the phosphate–leucine buffer and the guanidine hydrochloride solvents were determined pycnometrically.

Results

Genetic and Functional Analysis of leu-4 Mutants. Data used in mapping the *leu*-4 cistron were obtained from crosses involving mutant genes inserted into the genetic background of strain 89601, the inositol-requiring strain from which all *leu*-4 mutants were obtained. Since the degree of homology that resulted from inbreeding could not be estimated and significant differences in recombination frequency were observed in crosses involving different markers, the map distances obtained are approximations at best. The *leu*-4 locus is approximately equidistant between *ad*-5 and *leu*-3, an interval of about twenty units long, in the left arm of linkage group I. Estimates of the distance between *leu*-4 and the mating-type locus vary between one and five units. The recombination frequency between *leu*-4 and R83R-1-1-271, an allele of 35001 at the *cys*-5 locus (N. Murray, personal communication), is very low; out of 370 exchanges selected for in the interval *leu*-4–*ad*-5, only one involved recombination in the *leu*-4–*cys*-5 interval. The data obtained are consistent with the following order: *leu*-3, *leu*-4, *cys*-5, mating type, *ad*-5, centromere. The assignment of *leu*-4 distal to *cys*-5 is tentative, at best, for it is based on the segregation of the parental combination of *cys*-5 and mating type in a single segregant in which *leu*-4 and *cys*-5 had recombined.

A study of the growth responses of heterokaryons formed from all pairwise combinations of 118 *leu*-4 alleles, 49 of which responded prototrophically in one or more combinations, yielded the complementation map presented in Figure 1. The responses are graphically arranged in a linear display using the simple mapping rule that mutants which fail to interact share an overlapping defect (Catcheside and Overton, 1958). The only exception to linearity noted resulted from the fact that R142 is active in combination with R51. Two important features of the map should be noted. First,

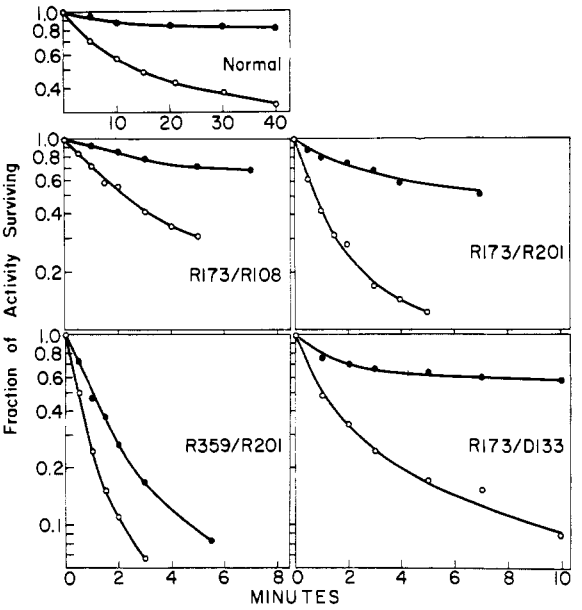

FIGURE 2: Thermal inactivation of the normal and hybrid α-isopropylmalate synthetase at 45°, pH 6.5. The protein concentrations of each preparation were adjusted to 1.2 mg/ml. The fraction of activity surviving is plotted as a function of time in the presence (●) and absence (○) of 2×10^{-4} M L-leucine.

most of the complementing *leu*-4 mutants obtained map terminally and most of the complexity of the map results from the behavior of unique mutants.

The genetic fine structure of the *leu*-4 cistron has not been determined with precision. Crosses between *leu*-4 mutants are relatively infertile and in many cases there is a strong selection against recovery of *leu*-4 segregants. However, the data obtained, although crude, are consistent with a reasonable degree of collinearity between the complementation and genetic maps.

Enzymology of Hybrid α-Isopropylmalate Synthetases. The ability of several *leu*-4 mutants (as triple mutants: *leu*-4, *leu*-1, and either *pan* or *inos*) to produce α-isopropylmalate synthetase when grown under conditions of derepression either as homokaryons or in heterokaryotic combination was determined, and the results are presented in Table I. Two mutants, R173 and D185, were able to produce trace amounts of active enzyme, but only D185 was able to grow, albeit only slightly in the absence of leucine. All heterokaryons yielded substantial amounts of synthetase. The best, R173/R201, yielded approximately 15% of that expected from a *leu*-1 mutant (the *leu*-1 cistron determines the structure of β-isopropylmalate dehydrogenase) grown under conditions of maximal derepression of synthetase synthesis.

Each of the hybrid enzymes behaved differently from the normal enzyme and from each other during purification. In general all of the hybrids are much less stable than the normal enzyme and present serious handling problems during purification. These studies were limited

TABLE I: The Specific Activity of α-Isopropylmalate Synthetase in Extracts of *leu*-4 Mutants and Heterokaryons.

Mutant[a]	Heterokaryon	Specific Activity[b]
R173(173)	—	>0.02
R359(353)	—	0
D133(133)	—	0
R108(108)	—	0
R201(31)	—	0
D185(185)	—	>0.02
—	R173/D133	9.8
—	R173/R108	19.6
—	R173/R201	23.8
—	R359/D133	5.96
—	R359/R108	6.1
—	R359/R201	5.4
—	R359/D185	2.83

[a] The number in parentheses denotes the complementation group to which the mutant belongs (see legend of Figure 1). [b] Specific activity was measured on 0–75% ammonium sulfate precipitates of crude extracts from which nucleic acid had been removed by protamine sulfate and particulate matter by centrifugation at 100,000*g* for 90 min.

therefore only to those heterokaryons which produced synthetase relatively efficiently.

The kinetics of heat inactivation of the four hybrid synthetases are presented in Figure 2. All are markedly more heat labile than the normal enzyme and all are considerably protected by leucine from thermal inactivation. Surprisingly, thermal inactivation kinetics easily resolved differences between the three R173 hybrid enzymes as well as the two R201 hybrid enzymes. Thus, in the hybrid enzymes studied here, thermal sensitivity probably reflects specific structural alterations associated with amino acid substitutions introduced by each of the *leu*-4 alleles.

Table II contains a comparison of the K_m values of the enzymes for each of the substrates as well as comparison of concentrations of leucine required for 50% inhibition of enzymatic activity. In each case the K_m determined for α-ketoisovalerate (α-KIV) was normal. The K_m values of the hybrid enzymes for acetyl coenzyme A (CoA) varied from normal, as in the case of R173/R108, to quite significantly more favorable for catalysis in the case of R359/R201. In addition to the lower K_m for acetyl CoA demonstrated by R359/R201, the hybrid displayed a greatly increased resistance to leucine inhibition. The correlation between the decrease in the K_m for acetyl CoA and resistance to leucine inhibition was pointed out in the preceding paper (Webster and Gross, 1965) with regard to changes in substrate binding exhibited by the leucine-resistant enzyme from FLR_{92}. In that case the change in K_m was small relative to the change in sensitivity to leucine inhibition. In the R359/R201 hybrid a more extensive change in the K_m for acetyl CoA is associated with a smaller change in sensitivity to leucine inhibition (FLR_{92} was completely resistant to leucine while the concentration of leucine required to inhibit 50% of the R359/R201 synthetase activity was about four times that required to inhibit wild type). The pH dependence of leucine inhibition of the R359/R201 hybrid synthetase is essentially the same as that of the normal enzyme, indicating that the change in sensitivity is probably not associated with the alteration of an ionizable group at or near the leucine binding site.

The kinetics of leucine inhibition of normal and hybrid synthetases at pH 7.5 are presented in Figure 3. In contrast to the behavior of the normal enzyme which is competitively inhibited by leucine at pH 7.5, all of the hybrid enzymes at pH 7.5 yield the mixed type of inhibition displayed by the normal enzyme at lower pH

TABLE II: The K_m Values and Leucine Concentrations Required for 50% Inhibition Displayed by Normal and Hybrid Synthetases.[a]

Heterokaryon	$K_m \times 10^{-5}$ M		Leucine Required for 50% Inhibition $\times 10^{-4}$ M
	Acetyl CoA	α-KIV	
Normal	2.45 ± 0.22 (7)	0.99 ± 0.07 (5)	1.4 ± 0.42 (5)
R359/R201	1.35 ± 0.13 (3)	1.20 ± 0.27 (3)	5.8 ± 0.52 (3)
R173/R201	1.9 ± 0.50 (2)	1.00 (1)	1.4 ± 0.10 (2)
R173/R108	2.4 ± 0.43 (4)	0.81 ± 0.42 (2)	2.05 ± 0.46 (2)
R173/D133	2.0 ± 0.56 (3)	0.90 ± 0.30 (2)	2.45 ± 0.21 (2)

[a] In each case the K_m was determined from Lineweaver–Burk plots of velocity at at least six different substrate concentrations. The number in parentheses refers to the number of independent determinations for both K_m and 50% inhibition. The NEM assay was used throughout. When α-KIV concentration was varied, acetyl CoA was held constant at 0.12 μmole/reaction mixture; when acetyl CoA concentration was varied, α-KIV was held constant at 0.5 μmole/reaction mixture. Leucine inhibition was measured in the presence of 3×10^{-5} M acetyl CoA.

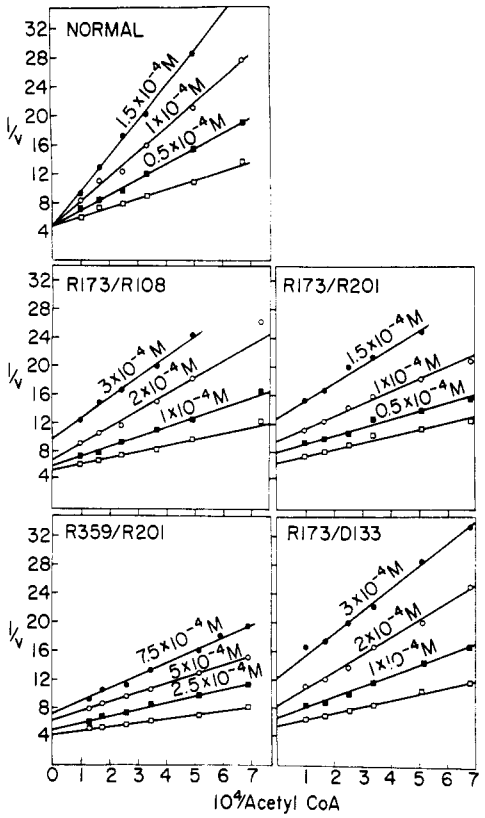

FIGURE 3: Lineweaver–Burk plots at pH 7.5 of the reaction velocity of the normal and hybrid α-isopropylmalate synthetases as a function of acetyl CoA concentration at several concentrations of L-leucine. The concentration of L-leucine employed for each set of determinations is listed above the corresponding line. Each reaction mixture contained 0.5 μmole of α-KIV.

values (Webster and Gross, 1965). It seems as if the structural changes in the hybrid enzymes do not permit the enzyme to undergo efficiently a pH-dependent structural transition that prevents simultaneous binding of substrate and inhibitor. Thus, the major demonstrable difference between the kinetic behavior of the hybrid enzymes seems likely to be related to conformational changes associated with the accessibility of the inhibitor and substrate binding sites to inhibitor and substrate rather than changes at the binding sites *per se*.

To determine whether the alterations in thermal sensitivity and kinetic properties of the hybrid synthetases were reflected in some change in size or shape, the sedimentation velocity of each hybrid enzyme was compared with that of the normal enzyme in sucrose density gradients according to the method of Martin and Ames (1961) with bovine hemoglobin as an internal standard. The calculated sedimentation coefficients for the four extensively studied hybrid enzymes and two others, R359/R108 and R359/D133, are listed in Table III. None differs significantly from that of the normal enzyme.

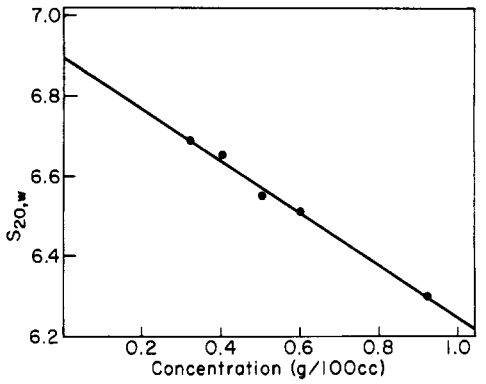

FIGURE 4: The determination of $s_{20,w}$ of α-isopropylmalate synthetase at zero protein concentration. Plotted are the empirical $s_{20,w}$ values obtained from determinations at different protein concentrations.

TABLE III: Sedimentation Coefficients of Normal and Hybrid Synthetases Determined by Sucrose Density Gradient Centrifugation.[a]

Heterokaryon	Average $s_{20,w}$ (S)	No. of Determn
Normal	6.68 ± 0.50	10
R173/R201	6.86	1
R173/R108	6.95 ± 0.1	2
R173/D133	6.25 ± 0.35	2
R359/R201	6.16 ± 0.13	2
R359/R108	6.49	1
R359/D133	6.98	1

[a] Centrifugation was carried out in sucrose gradients (5–20%) made up in 0.1 M phosphate buffer, pH 6.5, with 2×10^{-4} M leucine.

The Polymeric Nature of the Normal Synthetase. The observation that the hybrid enzymes behave quite differently from the normal enzyme, yet are of approximately the same size, raises the question as to whether complementation involves the aggregation of differently defective polypeptide subunits in the formation of a protein with at least the same general structural features of the normal enzyme. If polymerization of subunits were involved, we might expect the normal enzyme to consist of at least two identical polypeptide subunits. To determine whether or not the synthetase is a multimer, a sedimentation analysis of the normal enzyme was carried out by analytical ultracentrifugation in phosphate–leucine buffer and in buffer containing the dispersing agent guanidine hydrochloride.

Sedimentation velocity determinations of the purified normal synthetase as a function of protein concentration in 0.1 M phosphate buffer, pH 6.5, containing 2×10^{-4} M L-leucine (hereafter referred to as phosphate–

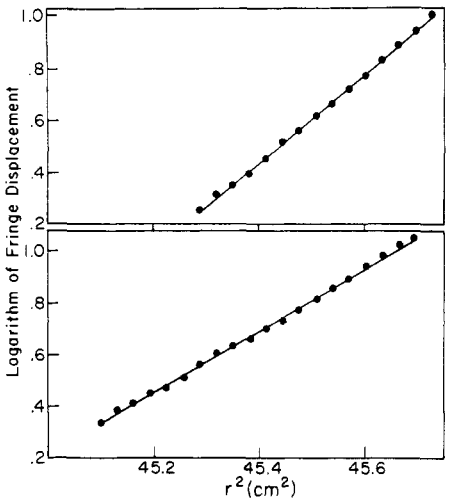

FIGURE 5: The logarithm of the vertical fringe displacement as a function of the square of the distance from the center of the rotor at equilibrium. The weight-average molecular weights derived from the slopes of the lines as described by Tanford (1961) are about 143,000 for the upper line and 48,000–50,000 for the lower line. The upper plot is that of data derived from centrifugation of α-isopropylmalate synthetase in phosphate–leucine buffer at a rotor speed of 21,740 rpm. The lower plot is that of data derived from centrifugation in 4 M guanidine hydrochloride and 0.1 M β-mercaptoethanol in 0.01 M Tris–HCl, pH 8.0, at a rotor speed of 33,450.

leucine buffer) yielded the data presented in Figure 4. An $s_{20,w}$ of 6.9 was obtained at zero protein concentration. The synthetase was also centrifuged in 6 M guanidine hydrochloride containing 0.1 M β-mercaptoethanol and 0.01 M Tris–HCl buffer, pH 8.0, at a concentration of 0.5 g/100 ml. This solvent served to disaggregate and to prevent reaggregation of the individual polypeptide chains, to break any disulfide bonds which might be present, and to prevent disulfide bond formation as a consequence of random reoxidation of sulfhydryl groups. A single sedimenting band with an $s_{20,w}$ of 1.6 was obtained. This $s_{20,w}$ is about the same as that obtained for γ-globulin subunits at the same concentration in the same solvent and corresponds to a weight-average molecular weight of about 43,000 for an unfolded polypeptide (Marler *et al.*, 1964). The $s_{20,w}$ of 6.9 obtained for the synthetase in phosphate–leucine buffer is consistent with a molecular weight of 115,000–155,000. The enzyme, therefore, seems to be made up of at least two, but more probably three subunits.

More accurate determinations of enzyme and subunit size were obtained by equilibrium centrifugation analysis. Molecular weight determinations on several preparations of the intact enzyme were carried out in phosphate–leucine buffer. The best preparation of synthetase used contained about 5–8% slightly heavier impurities as revealed by sedimentation velocity centrifugation. The sedimentation equilibrium centrifugation of this preparation did not reveal any significant heterogeneity and yielded an empirical weight-average molecular weight of about 143,000 (Figure 5). The relatively high speed of centrifugation and short height of the protein solution column might very easily have masked small amounts of impurity. However, the tendency of the protein to precipitate in the cell after long periods of centrifugation prevented analysis with longer columns at lower speeds. The fact that the preparation was known to contain a heavier impurity suggests that the real molecular weight might be somewhat less than the indicated weight-average molecular weight.

The sedimentation equilibrium of the synthetase was then determined in 4 M guanidine hydrochloride containing 0.1 M β-mercaptoethanol and 0.01 M Tris–HCl buffer, pH 8.0. The equilibrium plot shown in Figure 5 shows no appreciable heterogeneity and yields an empirical molecular weight of from 48,000 to 50,000.

The same preparation in 6 M guanidine hydrochloride–0.1 M β-mercaptoethanol was centrifuged at a lower rotor speed (21,740) with a longer solution column, thereby spreading out the material so that heterogeneity could be more easily detected. No curvature of the plot of the fringe displacement was observed over the initial four-fifths of the column, yielding a molecular weight of 43,000 for the major component. The remaining fifth curved upward, yielding a higher molecular weight of about 64,000. This upward shift can be accounted for by the suspected presence of a higher molecular weight impurity. These results suggest that the synthetase is made up of subunits each with a molecular weight of 43,000–50,000. Since the molecular weight of the synthetase is approximately 143,000, the enzyme is probably a trimer.

Concomitant with the sedimentation analysis of the synthetase, an amino acid and peptide analysis was undertaken to determine the number of polypeptide subunits involved and whether all were identical. The results of three separate analyses of the amino acid composition of the synthetase are presented in Table IV. The number of residues per molecule and the number per polypeptide subunit are presented. Calculations were based on the empirical weight-average molecular weight of 143,000 for the synthetase and the assumption that it is a trimer. The amino acid composition of the synthetase is not unusual for an acidic protein. It is interesting to note, however, that the enzyme contains 100 residues of leucine per molecule, although its synthesis and function are regulated as a function of leucine concentration. Of particular importance to structural analyses is the fact that the enzyme contains 150 arginine and lysine residues, and as expected contains relatively few methionine, cystine, tryptophan, and histidine residues.

The estimation of the total number of free thiol groups of the synthetase proved difficult. Titration at 25° in 0.1 M phosphate, pH 7.0, with PCMB by the procedure described by Boyer (1954) indicated the presence of at least ten easily titratable thiol groups per enzyme molecule. At least three more thiol groups were titrata-

TABLE IV: Amino Acid Composition of α-Isopropylmalate Synthetase.

Amino Acid	μmoles of Amino Acid/mg of Protein Prepn 1	2	3	Residues/ Molecule of 143,000 Mol Wt	Residues/ Monomer[a]
Lysine	0.536	0.558	0.573	79	26
Histidine	0.148	0.150	0.167	22	7
Arginine	0.478	0.504	0.516	71	24
Aspartic Acid	0.976	1.032	0.983	143	48
Threonine	0.468	0.464	0.456	66	22
Serine	0.552	0.589	0.571	82	27
Glutamic Acid	0.987	0.994	0.964	140	47
Proline	0.539	0.479	0.556	75	25
Glycine	0.653	0.706	0.673	97	32
Alanine	0.755	0.751	0.733	107	36
Valine	0.653	0.649	0.661	94	31
Methionine	0.114	0.110		16	5
Isoleucine	0.437	0.437	0.424	62	21
Leucine	0.705	0.705	0.689	100	33
Tyrosine	0.282	0.231	0.231	35	12
Phenylalanine	0.330	0.318	0.330	47	16
Half-cystine[b]			0.150	21	7
Tryptophan[c]			0.126	18	6

[a] Assuming the enzyme is a trimer; see text. [b] Determined as cysteic acid after performic acid oxidation. [c] Determinations by the spectrophotometric method and the two colorimetric procedures yielded 0.128, 0.120, and 0.130 μmole/mg protein of tryptophan, respectively. The average of the three determinations is listed.

ble if allowed to react with a twofold excess of PCMB for 24 hr, but precipitation after prolonged incubation interfered with more extensive titration. It seems quite possible then that the synthetase has very few disulfide bridges. If this is true, it is quite surprising that *N*-ethylmaleimide (NEM), which reacts with free thiol groups, has little effect on enzyme catalysis. It should be pointed out that 3-carboxy-4-nitrophenyl disulfide, a thiol exchange reagent, does inhibit the enzyme.

Figure 6 is an illustration of fingerprints of tryptic digests of the synthetase. The peptide pattern indicates that the synthetase contains a large number of neutral tryptic peptides. Even though the two-dimensional distribution of peptides was less than ideal under the conditions of electrophoresis and chromatography employed, peptide resolution was sufficiently sharp and reproducible to permit confident enumeration of ninhydrin-positive spots. The arginine and lysine content of the synthetase suggests that 150 peptides should be obtained upon tryptic digestion if the molecule were composed of either a single polypeptide chain or two or more different polypeptides. In actuality only 45 peptides were obtained, or approximately one-third of the number expected on the basis of the amino acid composition.

The limited amount of pure protein available precluded a concerted attempt to improve fingerprint resolution in order to obtain more reliable enumeration of peptides. Instead, because of the ease and reliability with which histidine-containing peptides can be identified, the fingerprints were sprayed with diazotized sulfanilic acid and the histidine-containing peptides were identified and enumerated. Ninhydrin-positive spots 6, 13, 15, 17, 18, and 35 contained histidine. The reaction at spot 18 was considerably more intense than at others, suggesting that this peptide may contain two histidine residues. Only six histidine-positive spots were discernible out of the 22 which might have been expected if the protein were composed of a single polypeptide chain or subunits differing in structure. The recovery of approximately one-third of the expected number of tryptic peptides and about one-third of the expected number of peptides containing histidine in addition to the molecular weight determinations of the protein and its subunits strongly suggests that the synthetase is made up of three identical subunits.

Discussion

The data presented indicate that α-isopropylmalate synthetase is a polymeric protein with a molecular weight of approximately 143,000, probably consisting of three identical subunits. None of the functionally active proteins obtained from complementation interactions in heterokaryons bearing different *leu*-4 mutations differs significantly in size or shape from the normal

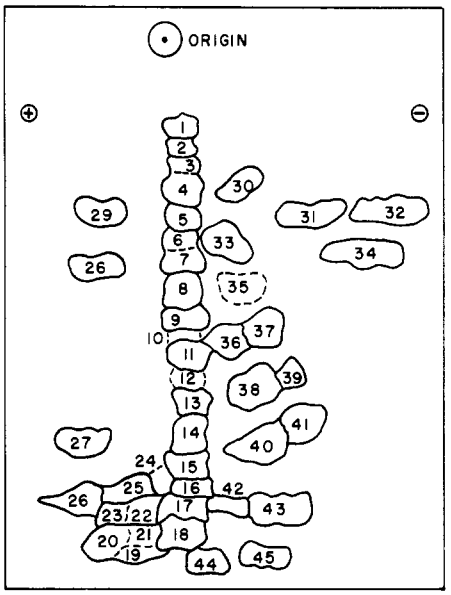

FIGURE 6: Tryptic peptide fingerprint of α-isopropylmalate synthetase. The peptides have been numbered arbitrarily. Dotted lines indicate poorly resolved peptides.

enzyme as determined by sedimentation velocity in sucrose density gradients. However, each of these enzymes differs from each other and from the normal enzyme rather specifically with respect to kinetic properties as well as thermal and inhibitor sensitivity. Thus, it seems highly unlikely to suppose that these catalytically active proteins are synthesized by aggregation of normal intact polypeptide subunits produced by some unique reshuffling process of incomplete polypeptide fragments. Rather, the evidence obtained at least overtly conforms with the large body of information accumulated for several different proteins (Schlesinger, 1964; Fincham and Coddington, 1963), indicating that effective complementation results from the polymerization of differently defective subunits yielding a catalytically active heteropolymer (Crick and Orgel, 1964). According to this view the individual deleterious alterations in the structure of the monomer can be compensated for by a normal region of another differently altered monomer in a heteropolymer. Thus, each of the hybrid enzymes is active catalytically as a function of a unique set of compensatory structural alterations which affect catalytic as well as structural properties without necessarily producing a great change in size or shape.

It seems important to iterate that this study was limited to those heterokaryons producing relatively large amounts of synthetase activity. Perhaps it is not too surprising then to find that the general catalytic properties of the hybrid enzymes studied are not too different from those of the normal enzyme. However, the hybrids were found to differ from each other and the wild type in those properties that can be presumed to involve relatively subtle conformational alterations. For example, in no case was substrate binding found to be adversely affected. The α-KIV binding constants displayed by the hybrid enzymes did not deviate significantly from that of the normal enzyme, and the acetyl CoA binding constants of the four hybrid enzymes were at least as favorable for catalysis as the normal enzyme. Indeed, the K_m for acetyl CoA of the R359/R201 hybrid was considerably more favorable for catalysis than that of the normal enzyme. Since the number and size of the functional substrate binding groups on the protein are probably small relative to the size of the protein, it is not likely that mutational alterations of the sort reported here would involve amino acid substitutions at the critical binding "sites." Instead it seems more likely that the more favorable binding constants displayed by the hybrid enzymes result from structural changes that affect accessibility of substrate to binding site.

The notion of the involvement of binding-site accessibility is supported by two different but related observations regarding the allosteric inhibition exerted by leucine on the synthetase. As pointed out here and in the preceding paper (Webster and Gross, 1965) leucine behaves, at least formally, as a competitive inhibitor at pH 7.5 of acetyl CoA binding to the normal enzyme. Thus, leucine bound by the normal synthetase excludes simultaneous binding of acetyl CoA and *vice versa*. At lower pH values the inhibition of catalysis of the normal enzyme is of the mixed type which, according to the simplest assumptions, suggests that at low pH both substrate and inhibitor may have simultaneous access to their respective binding sites. All of the hybrid enzymes studied retained, to varying degrees, sensitivity to leucine inhibition. However, strikingly, the kinetics of leucine inhibition with regard to acetyl CoA displayed by each of the hybrid enzymes are mixed even at pH 7.5. Thus, although the leucine binding site has been retained by each of the hybrid enzymes, some restriction has been imposed on the structure to interfere with the exclusion of acetyl CoA. It would seem then that the compensatory alterations of hybrid structure probably impart a somewhat more open conformation to the trimer, allowing greater substrate accessibility to its binding site. In this regard it is interesting to note that the hybrid least sensitive to leucine inhibition has the most favorable K_m for acetyl CoA.

Further support for the notion that the amino acid alterations in the individual subunits are only incompletely compensated for in the functional hybrid is the general observation that all hybrid enzymes are much more sensitive to thermal inactivation than the normal enzyme. This has been found to be true not only for the synthetase but for β-galactosidase (Perrin, 1963) and isopropylmalate isomerase (Gross and Webster, 1963) as well.

Several biological features of the complementation interaction displayed by *leu*-4 mutants are worthy of special note. Little or no correlation between leakiness and complementation efficiency has been observed. In fact many of the mutants which were capable of pro-

ducing significant amounts of enzyme failed to complement at all.

Although *leu*-4 mutants can be arranged almost without exception in a linear array on the basis of pairwise complementation interactions, most of the complexity of the map results from the complementation behavior of unique mutants covering many complementation groups. The distribution of mutants throughout the map is extremely bimodal, most of them mapping at or near either end. In general heterokaryons formed between mutants from opposite ends of the map produce more enzyme and grow better than heterokaryons formed between mutants located close together. These observations can be interpreted to mean that there may be only two primary regions of the synthetase where compensatory conformational alterations can occur with relative ease.

Acknowledgments

We wish to thank Drs. Robert Hill, Connie Harris, and Ralph Bradshaw for their help in the structural analysis of the enzyme.

References

Anson, M. L. (1941), *J. Gen. Physiol. 24*, 399.
Bencze, W. L., and Schmid, K. (1957), *Anal. Chem. 29*, 1193.
Block, R. J. (1951), *Arch. Biochem. Biophys. 31*, 266.
Boyer, P. D. (1954), *J. Am. Chem. Soc. 76*, 4331.
Bücher, T. (1947), *Biochim. Biophys. Acta 1*, 292.
Catcheside, D. G., and Overton, A. (1958), *Cold Spring Harbor Symp. Quant. Biol. 23*, 137.
Crick, F. H. C., and Orgel, L. E. (1964), *J. Mol. Biol. 8*, 161.
Fincham, J. R. S., and Coddington, A. (1963), *Cold Spring Harbor Symp. Quant. Biol. 28*, 517.
Gross, S. R., and Gross, H. S. (1961), *Genetics 46*, 868.
Gross, S. R., and Webster, R. E. (1963), *Cold Spring Harbor Symp. Quant. Biol. 28*, 543.
Kanarek, L., Marler, E., Bradshaw, R. A., Fellows, R. E., and Hill, R. L. (1964), *J. Biol. Chem. 239*, 4207.
Kielley, W. W., and Harrington, V. F. (1960), *Biochim. Biophys. Acta 41*, 401.
Lester, H. E., and Gross, S. R. (1959), *Science 129*, 572.
Levy, A. L., and Chung, D. (1953), *Anal. Chem. 25*, 396.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.
Marler, E., and Tanford, C. (1964), *J. Biol. Chem. 239*, 4217.
Marler, E., Nelson, C. A., and Tanford, C. (1964), *Biochemistry 3*, 279.
Martin, R. G., and Ames, B. N. (1961), *J. Biol. Chem. 236*, 1372.
McMeekin, T. L., Groves, M. L., and Hipp, N. J. (1949), *J. Am. Chem. Soc. 71*, 3298.
Moore, S. (1963), *J. Biol. Chem. 238*, 235.
Murphy, J. B., and Kies, M. W. (1960), *Biochim. Biophys. Acta 45*, 382.
Perrin, D. (1963), *Cold Spring Harbor Symp. Quant. Biol. 28*, 529.
Schlesinger, M. J. (1964), *Brookhaven Symp. Biol. 17*, 66.
Schlesinger, M. J., and Levinthal, C. (1963), *J. Mol. Biol. 7*, 1.
Spackman, D. H., Stein, W. H., and Moore, S. (1958), *Anal. Chem. 30*, 1190.
Spies, J. R., and Chambers, D. (1948), *Anal. Chem. 20*, 30.
Spies, J. R., and Chambers, D. C. (1949), *Anal. Chem. 21*, 1249.
Tanford, C. (1961), Physical Chemistry of Macromolecules, New York, Wiley, p. 260.
Warburg, O., and Christian, W. (1941), *Biochem. Z. 310*, 384.
Webster, R. E., and Gross, S. R. (1965), *Biochemistry 4*, 2309 (this issue; preceding paper).
Yphantis, D. A. (1964), *Biochemistry 3*, 297.