Am J Hum Genet 39:38–51, 1986

Argininosuccinate Lyase Deficiency: Evidence for Heterogeneous Structural Gene Mutations by Immunoblotting

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SUMMARY

Argininosuccinate lyase (AS lyase) deficiency is an inborn error of the urea cycle with extensive clinical and genetic heterogeneity. We investigated the biochemical basis of the enzyme defect and the genetic heterogeneity in this disorder using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting of fibroblast extracts. The AS lyase monomer in control fibroblasts was present in two bands of ~51 and ~49 Kd. Each of 28 mutant strains had some cross-reactive material (CRM) of the lower (~49 Kd) MW, in quantities ranging from trace to substantial levels. The ~51 Kd band was found in only six mutants with near-normal amounts of AS lyase CRM or high residual enzyme activity. The residual AS lyase enzyme activity in a mutant did not necessarily reflect the amount of the 49–51 Kd monomer in that strain. In contrast, there was a strong general correlation between the quantity of 49–51 Kd CRM in a mutant and the frequency of complementation by that mutant. In addition to the CRM of normal molecular weight (MW) (49–51 Kd), the majority of mutants (but not controls) had significant CRM present in one to five bands of MW < 49 Kd. The immunoprecipitation of at least one of these low MW bands was inhibited by purified human AS lyase. Mutants indistinguishable by clinical, enzymatic, or complementation analysis have been shown to be heterogeneous in their content of AS lyase CRM, greatly extending the number of distinct mutant alleles identified at this locus. These data demonstrate that multiple unique mutations in the structural gene coding for the monomer cause AS

Received November 20, 1985; revised February 12, 1986.

This work was presented in part at the Annual Meeting of The Society for Pediatric Research, San Francisco, 1984. This research was supported by grants from the Medical Research Council of Canada (to R. R. M.) R. R. M. is a Queen Elizabeth II Scholar and L. S. is a Medical Research Council Fellow. Address correspondence to R. R. M.

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lyase deficiency and that the AS lyase monomers made by these mutants may be unstable. Integration of these findings with enzymatic and complementation data has indicated the functional domain of the AS lyase monomer likely to be altered in certain mutants.

INTRODUCTION

Argininosuccinate lyase deficiency (argininosuccinic aciduria) is an autosomal recessive inborn error of the urea cycle characterized by significant clinical and genetic heterogeneity [1, 2]. The clinical heterogeneity is manifest by variation in the age of onset and severity of symptoms, so that patients are classified into neonatal (NN), subacute (SA), or late-onset (LO) groups [1]. The genetic heterogeneity is apparent from the extensive interallelic complementation identified by a complementation analysis of mutant fibroblasts from 28 patients [2]. Although all 28 mutants map to a single major complementation group, they are distributed among 12 subgroups, indicating that at least 12 allelic mutations at a single locus produce this disease. In microorganisms, interallelic complementation has been invariably found at loci coding for homomultimeric proteins [3]. Since argininosuccinic acid lyase (AS lyase) is a homotetramer (subunit MW 49,500 [4, 5]), the affected locus in argininosuccinic aciduria is likely to be the structural gene coding for the enzyme.

Very little is known about the biochemical and genetic basis of the enzyme deficiency or of the clinical and genetic heterogeneity. On the basis of complementation behavior, however, some predictions may be made about the nature of the affected protein, which we hypothesize to be the AS lyase monomer, in certain mutants [2]. In the 20 strains capable of complementation, the monomer must be present and able to aggregate with or conformationally correct other mutant monomers. The three mutants that are most capable of complementation (the frequent complementers) must be relatively normal in parts of the polypeptide involved in subunit interaction and are the strains most likely to have abundant quantities of the monomer. In contrast, noncomplementing mutants (eight strains) may fail to produce the monomer or may make a polypeptide so abnormal that it is unstable or incapable of associating with other monomers. However, three of these noncomplementing strains have high residual enzyme activity and are derived from three of the most mildly affected patients. The failure to detect complementation in tests with these three mutants is likely to reflect the inability of any other mutant monomer to improve on the already high in situ residual enzyme activity of these strains [2]. This high in situ activity indicates that these strains must synthesize an AS lyase polypeptide, perhaps in relatively abundant quantities.

Here, we sought evidence for mutation in the AS lyase structural gene as the cause of argininosuccinic aciduria, using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting. All 28 mutant strains had some AS lyase CRM at the MW (49–51 Kd) of the normal mono-

mer, in quantities ranging from trace to near-normal levels. However, the residual enzyme activity in a specific mutant did not necessarily reflect the quantity of the normal-sized monomer in that mutant. This observation is in contrast to the parallel changes in the amount and activity of an enzyme that would result from different mutations in a regulatory gene and is therefore direct evidence for mutation in the AS lyase structural gene in this disease. Interestingly, most mutants (but no controls) also contained substantial CRM with mobilities of lower apparent MW than the normal polypeptide. The immunoprecipitation of at least one of these bands was inhibited by purified human AS lyase, providing preliminary evidence that the AS lyase monomer synthesized in these mutants is unstable. We have also examined the quantity and MW of the lyase monomer in the mutants in terms of the clinical, enzymatic, and complementation phenotypes of each mutant. The strains most capable of complementation, or with high residual enzyme activity, had near normal amounts of CRM of the normal size. Other mutants, indistinguishable from each other by clinical, enzymatic, or complementation analysis, have been clearly shown to be heterogeneous by immunoblotting, extending the number of mutant alleles identified at this locus.

MATERIALS AND METHODS

Fibroblasts

Skin fibroblast strains were derived from 28 unrelated patients with AS lyase deficiency [2] and cultured without antibiotics in α-minimal essential medium [6] supplemented with 10%–15% fetal bovine serum. All strains were free of mycoplasma as determined by direct culture and by a uridine phosphorylase assay.

Materials

Acrylamide, bisacrylamide, sodium dodecyl sulphate (SDS), and nitrocellulose (pore size 0.45 micron) were obtained from BioRad (Mississauga, Ontario), [^{14}C]-labeled protein markers from Amersham (Oakville, Ontario), and [^{125}I]protein A from New England Nuclear (Lachine, Quebec). Other reagents were of the highest purity grade and came from Sigma (St. Louis, Mo.) or Fisher Scientific Co. (Fairlawn, N.J.).

Preparation of Crude Cell Lysates

Cells from a single 100-mm culture dish were detached with 0.1% trypsin in citrate saline containing 0.1% glucose. The resulting cell pellet was washed twice with phosphate-buffered saline (PBS) at 4°C and resuspended in lysis buffer (0.1 M Na_2HPO_4, pH 7.2, 0.1 mM phenylmethyl sulfonyl fluoride [PMSF], 5×10^{-4} M DL-dithiothreitol [DTT], 1% Triton X-100) at a cell concentration of 8×10^7 cells/ml. Following lysis, cell debris was removed by centrifugation for 30 min at 4°C (Brinkmann Eppendorf Centrifuge 3200) and the supernatant collected. Mouse and human liver were homogenized in the 0.1 M Na_2HPO_4 buffer, and a high-speed supernatant was prepared for electrophoresis as described below. The protein concentration of crude cell lysates was determined by the method of Lowry et al. [7], using bovine serum albumin as the standard.

Polyacrylamide Gel Electrophoresis in the Presence of SDS

Proteins were separated on 9.5% polyacrylamide gels (15.5 cm × 13 cm × 0.15 cm) using the SDS-PAGE buffer system of Laemmli [8]. Crude fibroblast lysates and liver

homogenates were prepared for SDS-PAGE by solubilization in 3% (v/v)SDS, 1% (v/v)glycerol, 0.7 M 2-mercaptoethanol, and 0.01% bromophenol blue in 0.06 M Tris, pH 6.8. Solubilized mixtures containing either 300 μg of fibroblast lysate protein, 10 μg mouse liver, or 30 μg human liver homogenate protein were incubated for 3 min at 100°C. Electrophoresis was in 25 mM Tris, 192 mM glycine, 1% SDS reservoir buffer at a constant current of 16 mA until the tracking dye was within 1 cm of the bottom of the separating gel.

Electrophoretic Transfer of Protein to Nitrocellulose

Protein transfer from slab gels to nitrocellulose sheets, using the BioRad Trans-Blot™ cell and Model 160/1.6 Power Supply, was performed as described by Towbin et al. [9]. The transfer was carried out for 22 hrs at 8 V/cm, at 4°C.

Immunological Detection of AS Lyase

Following protein transfer, nonspecific binding sites on the nitrocellulose were blocked by soaking the sheets in 50 ml of 3% bovine serum albumin (BSA), 0.9% NaCl, 10 mM Tris buffer, pH 7.4, for 1 hr at 37°C on a rocker platform. A rabbit monospecific anti-AS lyase antiserum, raised against human hepatic AS lyase and described previously [4], was then added at a ratio of 1:200 in 150 mM NaCl, 20 mM Na_2HPO_4, pH 7.3, 3% BSA, 0.02% NaN_3. The nitrocellulose sheets were incubated with antibody for 4 hrs with gentle shaking at room temperature and then washed five times over 75 min with 50 ml of washing buffer (150 mM NaCl, 20 mM Na_2HPO_4, pH 7.3, 0.25% Triton X-100) per wash.

Antigen-antibody complexes on the nitrocellulose were detected by incubating the sheets with 4.5×10^6 cpm [^{125}I]protein A in 25 ml washing buffer for 5 hrs at room temperature. The sheets were then washed overnight in 100 ml washing buffer followed by six 50 ml changes over 1.5 hrs and allowed to dry.

Affinity-Purified Anti-AS Lyase Antisera

Anti-AS lyase antisera was purified by affinity chromatography using pure human hepatic AS lyase prepared as described [4]. The AS lyase (4.5 mg) was coupled to 1 g of CNBr-activated Sepharose 4B, as described by the manufacturers (Pharmacia, Piscataway, N.J.). The AS lyase-Sepharose, packed in a 0.9 × 3-cm column, was equilibrated with 1 liter of PBS (0.15 M NaCl, 0.01 M Na_2HPO_4, pH 7.2) and 5 ml of anti-AS lyase antisera applied to the column. The effluent was recirculated onto the column at 0.8 ml/min for 2 hrs. After an overnight wash with 1 liter of PBS, 100 ml of 0.2 M KSCN was passed through the column followed by 0.17 M acetic acid to elute the antibodies. The acetic acid wash was collected in 1-ml aliquots, immediately neutralized with concentrated NH_4OH to a phenol red detected neutral pH, and buffered with 100 μl of 10× PBS. After an 18-hr dialysis, the protein-containing fractions were assayed for anti-AS lyase antibodies [4]. More than 90% of the anti-AS lyase antibody activity applied to the column was recovered.

Autoradiography

The nitrocellulose sheets were wrapped in Saran Wrap and exposed to Kodak X-Omat AR film (Eastman Kodak Co., Rochester, N.Y.) at −70°C using a Dupont Cronex Lightning-Plus Intensifying Screen (E.I. Dupont De Nemours, Wilmington, Del.). Exposure times were for 5–9 hrs, unless indicated otherwise in the figure legends.

[^{35}S]Methionine-labeling of Fibroblast Cultures

Radioactive labeling of cells was performed as described by Robinson et al. [10], with modifications. Fibroblasts were grown to early confluence in 150-cm^2 tissue-culture

flasks, two flasks per experiment. Forty-five minutes prior to radioactive labeling, the medium was removed from the flasks and replaced by 10 ml of α-medium containing 10 μM methionine (final concentration) and 15% dialyzed fetal calf serum. The labeling of the cells was initiated by replacement of the medium with 5 ml of the same medium containing 200 μCi/ml of [^{35}S]methionine (100–1200 Ci/nmol, New England Nuclear, Boston, Mass.). After a 16-hr incubation, the radioactive medium was removed and the flasks washed twice with HEPES-buffered saline. The cells were then lysed by incubation for 1 hr in 1.5 ml in a buffer containing 0.15 M NaCl, 30 mM HEPES, pH 7.3, 5 mM EDTA, 1 mM PMSF, and 1% Triton X-100 at 4°C, and harvested by scraping with a rubber policeman. The supernatant was mixed with an equal volume of CCl_4, vortexed, and centrifuged at 15,000 g for 10 min. The supernatant was then adjusted to 2% Triton X-100 and 0.4% SDS and incubated with 400 μl IgSorb (protein A fixed to staphylococcus aureus, 1.99 mg/ml, Enzyme Center, Boston, Mass.) for 2 hrs at 4°C. After centrifugation at 15,000 *g* for 10 min, the supernatants were incubated with 20 μl nonimmune serum overnight at 4°C, and followed by a 2-hr incubation with 200 μl IgSorb. After centrifugation for 10 min, supernatants were aliquoted into three fractions and incubated with either 2 μl nonimmune serum, 5 μl anti-AS lyase antiserum, or 5 μl preabsorbed anti-AS lyase antiserum for 2 hrs at 4°C. Preabsorption involved incubation of 5 μl antiserum with 36 μg pure human hepatic AS lyase protein for 2 hrs at 4°C. The above reactions were then incubated with 20 μl, 50 μl, and 50 μl IgSorb, respectively, for 2 hrs at 4°C. After centrifugation, pellets were washed three times with a high salt buffer (10 mM Tris-HCl, pH 7.5, 1% NP-40, 2 mM EDTA, 0.5 M NaCl, 1 mg/ml BSA) followed by three washes in a low salt buffer (10 mM Tris-HCl, pH 7.5, 1% NP-40, 150 mM NaCl, 2 mM EDTA). The final pellet was resuspended in 50 μl solubilizing buffer, boiled for 5 min, centrifuged for 5 min, and run on a 10% SDS-polyacrylamide gel overnight. The gel was fixed in 10% trichloroacetic acid, 30% methanol for 1 hr, reacted with Enhance (New England Nuclear) for 30 min, washed in H_2O for 45 min, and dried. The autoradiogram was exposed for 96 hrs.

RESULTS

AS Lyase CRM in Control Skin Fibroblasts and Liver

Initially, we determined the nature and quantity of AS lyase CRM in control strains and established the dependency of the immunoblot results on the use of the monospecific anti-AS lyase antisera. A major band at the expected size (49–51 Kd) of the AS lyase monomer was visualized in six control fibroblast strains and in extracts of mouse and human liver (fig. 1). The amount of CRM in different control strains was consistent within a range of about twofold and showed little variation in different blots of different lysates of the same strain.

Immunoblots of every fibroblast extract (control or mutant) also displayed a band of mild and relatively invariate intensity of ~74 Kd MW (e.g., fig. 1*A*). This band was not seen in liver extracts (fig. 1*A*) or when nonspecific probes were used (fig. 1*B* and *C*); its identity remains to be determined.

The detection of all immunoblot bands was completely dependent on the use of the anti-AS lyase monospecific antisera. No bands were observed when the protein blots were probed with nonimmune serum and [^{125}I]protein A (fig. 1*B*) or with [^{125}I]protein A alone (fig. 1*C*). In addition, the 49–51 Kd band was also detected by anti-AS lyase antisera that had been affinity-purified (data not shown for controls; mutants are illustrated in fig. 5). The specificity of the AS lyase monomer band was also demonstrated by immunocompetition experiments using pure AS lyase, described below.

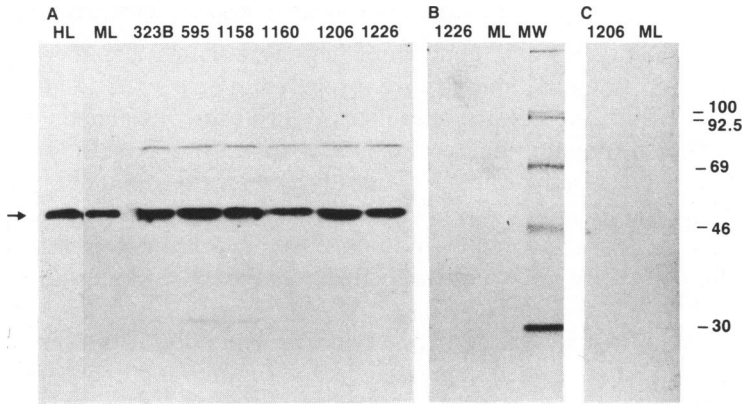

FIG. 1.—AS lyase immunoblot analysis of human liver (HL), mouse liver (ML), and six control fibroblast strains (numbered 323B–1226). Crude cell lysates (30 μg of protein for HL, 10 μg for ML, and 300 μg for fibroblasts) were subjected to SDS-PAGE and electrophoretic transfer to nitrocellulose filters as described in MATERIALS AND METHODS. The filters were treated as follows. *A*, anti-AS lyase antiserum and [^{125}I]protein A. *B*, nonimmune serum and [^{125}I]protein A. *C*, [^{125}I]protein A alone. MW = [^{14}C]-labeled protein molecular weight standards. *The arrow on the left* indicates the position of the AS lyase band, MW ~49–51 Kd.

In experiments in which the amount of cell protein subject to electrophoresis was varied, the 49–51 Kd band could be resolved into distinct bands of ~49 and ~51 Kd (fig. 2). This distinction could be made for 150–250 μg of applied protein but was less evident with loadings of 300 μg. As the amount of cell protein per lane was decreased below 150 μg, only the ~51 Kd band is seen, indicating that it is the predominant species. Recent studies of human AS lyase cDNA have determined that the ~51 Kd polypeptide is the primary translation product [11], suggesting that the ~49 Kd band is a proteolytic product found in normal cells.

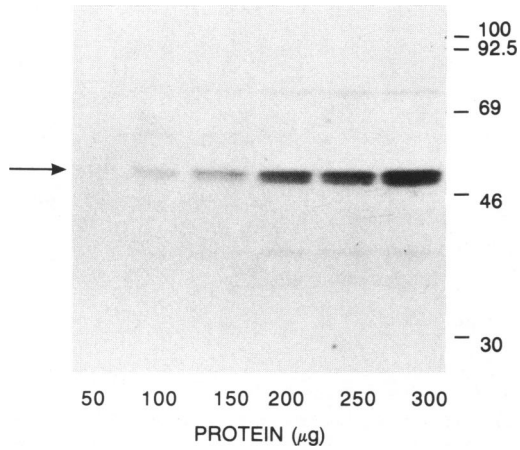

FIG. 2.—AS lyase immunoblot analysis as a function of increasing amounts (50–300 μg protein) of a control fibroblast crude cell lysate. The methods were those described for figure 1. *The arrow* indicates the position of the AS-lyase band of MW ~51 Kd.

AS Lyase CRM of Normal MW (~49–51 Kd) in Mutant Fibroblasts

The immunoblots of eight noncomplementing mutants and the three most frequently complementing mutants are displayed in figure 3. Ten mutants constituting four complementation subgroups [2] (arbitrarily designated here as A, B, C, and D) are shown in figure 4. Control strain 1160 in figure 4, run in the same immunoblot as subgroup C, is displayed with these mutants to demonstrate directly the reduction in CRM in these mutants. A summary of the data from these 21 strains is presented in table 1. The data from seven mutants, for which the results were not significantly different from the 21 strains presented, are not shown.

All 28 lyase mutants exhibited band patterns generally distinct from normal cells. Each mutant contained the ~49 Kd band, but the relative amount of this band varied from trace (e.g., strain 1181 and 1041, fig. 4) to substantial levels (e.g., strains 1078 and 1017, fig. 3). Interestingly, only six of the mutant strains contained the ~51 Kd band, and five of these strains were the mutants with the highest levels of CRM (strains 945 to 1254, fig. 3). As clearly shown in this figure, the absence of the higher MW band was not related to the inability to detect it in strains with low overall CRM. The ~51 Kd band could be seen as specifically absent in most of the low CRM strains (compare strain 928 showing the 49–51 Kd doublet to strains 1078 and 1017 with only the ~49 Kd band).

In the mutant population, the residual enzyme activity in a strain did not necessarily reflect the quantity of the normal MW (49–51 Kd) CRM present in that strain (fig. 3, table 1). In particular, a large quantity of CRM of normal MW did not always confer proportionately high residual enzyme activity on a strain (fig. 3, table 1). The quantity of the 49 Kd band varied from very small to substantial amounts in the other mutants (figs. 3 and 4).

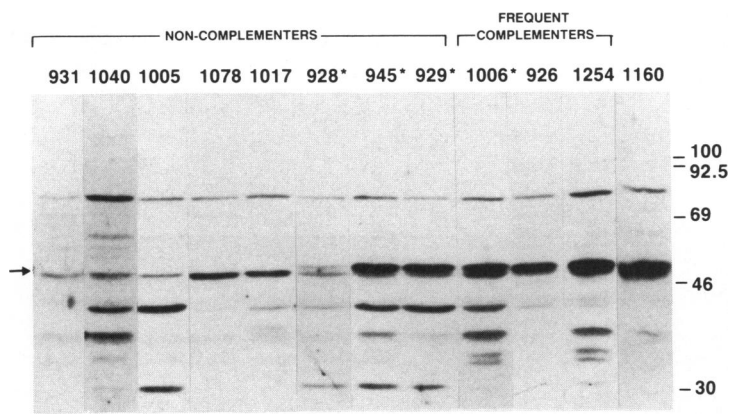

FIG. 3.—AS lyase immunoblots of noncomplementing and frequently complementing AS lyase-deficient fibroblast strains (numbered 931–1254) compared to a control strain (1160). Crude cell lysates (300 μg protein) of each strain were subjected to SDS-PAGE and immunoblotted as described in MATERIALS AND METHODS. The four strains with high residual enzyme activity, from mildly affected patients [2], are indicated by *an asterisk*. *The arrow on the left* indicates the position of the AS lyase band of MW ~49 Kd. The position of the molecular weight standards is indicated on *the right side of the figure*.

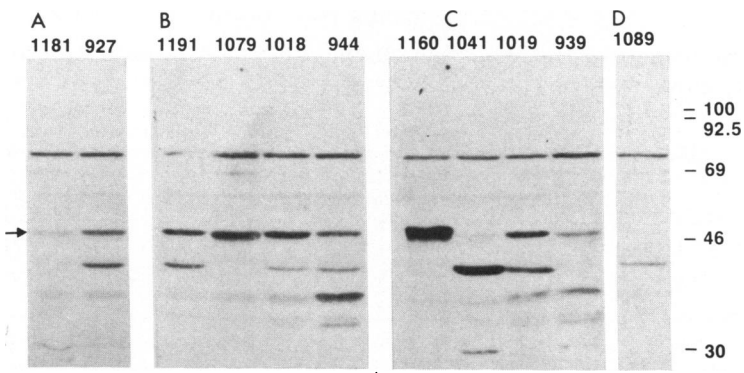

FIG. 4.—AS lyase immunoblots of AS lyase-deficient strains that are displayed according to the complementation subgroups (*A–D*) to which they belong [2]. These strains complemented with two other subgroups (*A:* 1181, 927, and *B:* 1191–944), or one other subgroup (*C:* 1041–939 and *D:* 1089). Strain 1160 is a control. Crude cell lysates (300 μg protein) of each strain were treated as described in the legend to figure 1. *The arrow* indicates the position of the AS lyase band of MW ~49 Kd. The position of the MW standards is indicated on *the right side of the figure.*

Although there was also no general relationship between the clinical phenotype (NN, SA, or LO group) of a strain and the quantity of 49–51 Kd CRM (table 1), three of the four mutants (928, 945, 929, and 1006) with high residual enzyme activity had substantial quantities of 49–51 Kd CRM.

There was a strong correlation between the quantity of 49–51 Kd CRM in a mutant and the frequency of complementation by that mutant. The three strains (1006, 926, and 1254) that participated in 90% of all the positive complementation tests [2] were members of the group of five highest CRM strains (fig. 3). Conversely, of the nine strains with the lowest amount of the normal MW monomer (designated by a single + in the last column of table 1; one strain is not listed), seven complemented only once or never, while only two complemented more frequently. This disparity in the distribution of low CRM mutants between poorly complementing and more frequently complementing strains (seven low CRM strains in 12 poor complementers vs. two low CRM strains in 16 better complementers; table 1) is highly significant ($\chi^2 = 4.5$, d.f. = 1, $P < .05$). Not unexpectedly, exceptions to this general correlation between the quantity of the 49–51 Kd monomer and complementation behavior were also found. For example, the noncomplementing mutants, 1078 and 1017 (fig. 3), had large quantities of ~49 Kd CRM.

The genetic heterogeneity amongst the AS lyase mutants, identified by complementation analysis [2], can be extended by comparison of the AS lyase CRM in the complementation subgroups in which there is more than one strain. For example, the three mutants comprising subgroup C (fig. 4; strain 1160 is a control) can be seen to differ greatly in both their content of the normal MW monomer as well as in their content and distribution of lower MW species (discussed below). Examination of the mutants in other complementation subgroups (e.g., A and B, fig. 3) indicates additional complex patterns of CRM.

TABLE 1

PHENOTYPIC PROPERTIES OF AS LYASE-DEFICIENT FIBROBLASTS FROM 21 PATIENTS

Strain	Clinical phenotype*	Frequency of complementation†	In situ residual enzyme activity‡	Quantity of CRM at 49–51 Kd§
Noncomplementers (high residual activity in situ)				
928	LO	0	62.0%	+
945	LO	0	34.1%	+ + +
929	LO	0	56.2%	+ + +
Noncomplementers (low residual activity in situ)				
931	NN	0	3.2%	+
1040	LO	0	0.6%	+
1005	SA	0	1.1%	+
1078	NN	0	0.9%	+ +
1017	NN	0	2.1%	+ +
Frequent complementers				
926	NN	5	1.4%	+ + +
1006	LO	5	15.0%	+ + +
1254	NN	6	3.0%	+ + +
Other complementing strains				
1181	NN	2	2.9%	+
927	LO	2	4.0%	+ +
1191	LO	2	3.2%	+ +
1079	LO	2	1.7%	+ +
1018	LO	2	2.0%	+ +
944	LO	2	1.2%	+ +
1041	NN	1	0.6%	+
1019	LO	1	0.9%	+ +
939	LO	1	1.6%	+
1089	LO	1	1.5%	+

* NN = neonatal onset, SA = subacute onset, LO = late onset.

† This figure is the no. other subgroups with which this strain complements [2].

‡ Residual activity is expressed as the percent of control cell incorporation of ^{14}C from L-[uriedo $-^{14}C$]citrulline and of ^{3}H from [4, $5-^{3}H$]leucine into acid-preciptable material, by intact attached cells. The data are from [2]. All mutants had < 3% of residual enzyme activity if the assay was done directly using a crude cell homogenate [2].

§ The amount of CRM of 49–51 Kd in the immunoblots of the mutant strains (figs. 3 and 4) is indicated in the last column as follows: + + + = CRM present in quantities similar to controls; + + = CRM present in quantities less than controls, but greater than the eight mutants with the lowest amount of CRM = +.

Low MW (< 49 Kd) AS Lyase CRM in Mutants

In addition to variation in the quantity of the AS lyase monomer, the second major abnormality in mutant cell extracts was the finding that they contained several species of CRM of lower apparent MW than the 49–51 Kd bands of the normal polypeptide. The occurrence and intensity of these bands were variable among different mutants, but they formed a discrete pattern of bands of MW ~42, 38, 35, 34, and 30 Kd. The larger of these bands (~42 and ~38 Kd) were

generally the most abundant as well as the most common (figs. 3 and 4). Close examination of the banding pattern of normal cell extracts occasionally revealed the same set of bands, but in very low quantities. Some mutants, notably strain 1006 (fig. 3) contained all five lower MW species, while others (strains 931, and 1078, fig. 3) were, like the control strains, essentially devoid of these bands. One other mutant with virtual absence of the lower bands, strain 1181 (fig. 4) had only trace quantities of the ~49 Kd and none of the ~51 Kd band. Of the mutants with complex band patterns, strains 1005 (fig. 3) and 1041 (fig. 4) were particularly unusual because they had substantial CRM in lower MW bands, yet little CRM of the normal MW (49–51 Kd). The overall distribution and abundance of the mutant banding patterns was generally reproducible between different cell lysates of a single strain.

The lower MW bands were shown to be AS-lyase CRM in two ways. First, an immunoblot of two mutants demonstrated that affinity-purified anti-AS lyase antisera (fig. 5*B*) detected the same lower MW bands recognized by untreated anti-AS lyase antisera (fig. 5*A*). Second, immunoprecipitation by anti-AS lyase antiserum of the ~49 and ~42 Kd [^{35}S]methionine-labeled proteins from a control and a mutant strain (fig. 6, lane 2 [control], lane 4 [mutant]) was prevented by purified human liver AS lyase [4] (fig. 6, lane 3 [control], lane 5 [mutant]).

The abundance and distribution of the lower MW bands in control or mutant strains was not affected by treatments designed to enhance or reduce proteolysis during or after cell harvest. The use of proteolytic inhibitors (0.1 mM PMSF, 5 mM *N*-ethylmaleimide, or 2.5 mM EDTA) in the homogenizing buf-

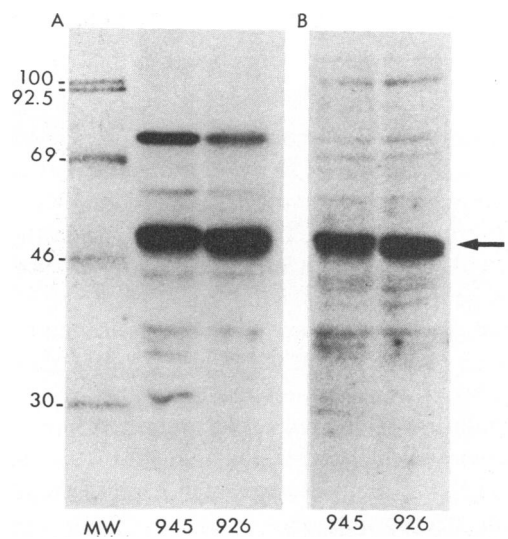

FIG. 5.—AS lyase immunoblots of two AS lyase-deficient strains (945, 926) done using untreated anti-AS lyase antisera (*A*) or affinity-purified anti-AS lyase antisera (*B*). Crude cell lysates of the two strains were treated as described in the legend to figure 1. *The arrow* indicates the position of the AS lyase band of MW ~49 Kd. The filter was exposed for 20 hrs in *A*, and for 96 hrs in *B*. The position of the MW standards is indicated on *the left side of the figure*.

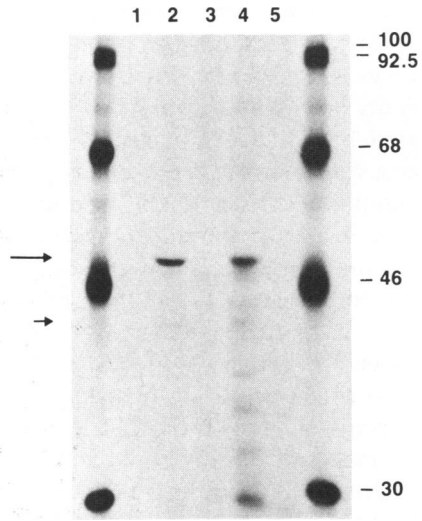

FIG. 6.—An SDS-polyacrylamide gel of immunoprecipitated [^{35}S]methionine-labeled protein from control human fibroblast strain 323B (*lanes 1, 2,* and *3*) and AS lyase-deficient strain 945 (*lanes 4* and *5*). Nonimmune serum was used in *lane 1*, anti-AS lyase antiserum in *lanes 2* and *4*, and anti-AS lyase antiserum preabsorbed with pure human AS lyase in *lanes 3* and *5*. *The longer arrow* indicates the position of the AS lyase band of MW ~49 Kd. *The shorter arrow* indicates the position of the band of MW ~42 Kd, detectable only in *lanes 2* and *4*. *The outside lanes* contain [^{14}C]-labeled protein molecular weight markers, whose size is indicated *on the right*.

fer, placement of the cells at 37°C for 2 hrs after trypsinization, or incubation of cell lysates at 37°C for 2 hrs after their preparation were all without effect (data not shown).

DISCUSSION

The major conclusions of this work are that human AS lyase deficiency results from mutations in the AS lyase structural gene and that a large number of distinct alleles are present in this mutant population. The principal evidence identifying the structural gene as the affected locus is that the quantity of CRM does not correlate with the amount of residual enzyme activity in individual mutants. Regulatory gene mutations, in contrast, would be expected to result in proportionate changes in these parameters. The presence of substantial quantities of CRM in lower MW bands in the majority of the mutants also suggests that the mutations affect the AS lyase structural gene and, as discussed below, that increased degradation of the unstable mutant AS lyase monomer is the result. Since we previously demonstrated that all of the mutants we studied affect a single locus [2], we now conclude that each of these strains has mutations in the AS lyase structural gene.

The extensive genetic heterogeneity in mutants at this locus is apparent from the wide variation in the amount and size of AS lyase CRM seen in the immunoblots. Heterogeneity in the quantity of CRM of normal molecular weight in different mutants is likely to result from heterogeneity in the different muta-

tions disrupting the AS lyase structural gene in each strain. Of the 12 complementation subgroups that we previously identified in this mutant population [2], at least three have been shown by immunoblot analysis to be heterogeneous in their content of AS lyase monomer of normal MW (49–51 Kd). The ability to compare the AS lyase CRM in the mutants within a specific complementation subgroup has therefore permitted a degree of analytical resolution that would not have been possible using immunoblotting or complementation analysis alone. These observations corroborate the extensive heterogeneity in the mutations at this locus and suggest that molecular analysis of some of these mutants will disclose the existence of many more alleles than those that have been recognized to date.

Our finding that AS lyase in normal fibroblasts consisted of both ~51 and ~49 Kd species was unexpected, since purified human liver AS lyase has an apparent MW in denaturing gels of ~49 Kd [4, 5]. However, we recently determined by sequence analysis of a full-length human AS lyase cDNA that the MW of the human AS lyase monomer is 51,663 [11]. A comparable size of 52,010 MW has been obtained for yeast AS lyase [12]. Consequently, the ~51 Kd band detected by immunoblotting of fibroblasts appears to be the full-length, unmodified, primary translation product; the enzymatically active ~49 Kd AS lyase monomer purified from human liver is likely to be a proteolytic product of the primary polypeptide.

The presence of substantial quantities of CRM in lower molecular weight bands is a significant property only of the mutant phenotype. This observation is strong evidence that these polypeptides are AS lyase derivatives. This assertion is supported by the detection of these bands by affinity-purified anti-AS lyase antibodies, the immunoprecipitation of the ~49 and 42 Kd bands by the antisera, the ability of purified AS lyase to block the immunoprecipitation of these two bands, and the fact that the mutations in these cells are in the AS lyase structural gene. However, the mechanism of formation of these lower MW AS lyase species is unknown. Since mutations in the structural gene have been shown to increase the degradation rate of other mutant enzymes [13, 14], we speculate that the abnormal quantities of the lower MW bands in these mutants result from an increased rate of degradation of the unstable mutant monomers. If this is correct, the 24 mutants with lower MW bands have abnormalities in the coding region of the AS lyase gene. The presence of these lower MW bands in control strains, albeit in trace quantities, is consistent with the hypothesis that these protein species are the products of a normal multistep proteolysis of the AS lyase monomer. Conversely, since none of the lower MW bands are unique to any one strain, there is no evidence that any of the mutations lead to the synthesis of truncated AS lyase polypeptides large enough to be detected by our system.

On the basis of the four phenotypic properties (subunit quantity, catalytic function, subunit interaction, and clinical group), which we examined in this and previous work [2], tentative conclusions can be made about the nature of the mutations present in some mutants. Since the majority of these mutants are likely to be genetic compounds [2, 15], these conclusions are correct only if the

two alleles are similar or if the observed properties derive predominantly from one of them (of the strains to be discussed, only 1254 is from a consanguineous mating). First, the identification of CRM in every mutant fulfills the prediction made by the high frequency of interallelic complementation in this mutant population: that the mutants capable of interallelic complementation must be able to synthesize the AS lyase monomer. If the presence of the lower MW bands does signify that most mutants synthesize labile monomers, then the high frequency of complementation in these strains may result from a common mechanism. If most of the mutants synthesize labile monomers, complementation between these mutants could result from stabilization of the defective monomers in the hybrid tetramer found within complementing heterokaryons. The increased stability of hybrid tetramers may result from the conformational correction of one mutant monomer by another, as proposed by Crick and Orgel [16].

Second, the mutations have been the least disruptive in four strains (928, 929, 945, 1006) from mildly affected patients; none of the properties of the AS lyase monomer have been entirely ablated in these cells. In three of these strains, the monomer is present in substantial quantity, and catalytic activity is relatively high even in the three strains that do not complement, indicating that subunit interaction must be comparatively intact. In addition, these four strains all share the property of having a ~51 Kd band, a feature found only in the two other mutants with high CRM (strains 926 and 1254) and controls. Since strain 928 is the only low CRM mutant with the ~51 Kd band, and also the only low CRM mutant with high residual enzyme activity, it is tempting to speculate that the ~51 Kd band is a necessary (although not sufficient) characteristic for high residual activity.

Third, the low enzyme activity in strains 926 and 1254 is likely to result from a mutation that interferes with the active site of the enzyme; both of those strains produce relatively stable monomers capable of tetramer formation, since they have abundant 49–51 Kd CRM and complement frequently. The correlation between CRM and complementation frequency that these strains illustrate is not unexpected, since mutations such as single base changes might drastically alter activity without affecting the protein structure.

Fourth, our finding that strains with subunits in very low abundance complemented less frequently is not surprising. These mutants may produce only small numbers of monomers (e.g., cis regulatory or splicing mutations) or the monomers may be highly unstable, even in the presence of more stable monomers in a tetramer. Exceptional strains such as 1181, which complement but have very low amounts of CRM (of any size), may contain alleles that are stabilized by the formation of hybrid tetramers with the monomers of other mutants.

In summary, we have demonstrated that multiple unique mutations of the AS lyase structural gene are the cause of human argininosuccinic aciduria. Correlation of the data on AS lyase CRM with the enzymatic, complementation, and clinical phenotypes has provided insight into the pathogenesis of the enzyme defect in each mutant. In addition, in some strains, it has been possible to

suggest the functional domain of AS lyase likely to be altered by the mutation. These findings give insight into the clinical and genetic heterogeneity of human AS lyase deficiency and provide a rational framework for the investigation of these mutants at the level of the gene.

ACKNOWLEDGMENTS

We thank Dr. J. Riordan for advice on immunoblotting, Drs. R. Gravel and H. Willard for reviewing the manuscript, Elizabeth Sexsmith for assistance, and Shirley Sauve for typing the manuscript.

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