Reversion of a Chinese Hamster Cell Auxotrophic Mutant

(serine hydroxymethyltransferase/isozymes/somatic cell genetics/glycine)

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ABSTRACT A mutant cell strain derived from a Chinese hamster line by mutagenesis with ethyl methane sulfonate requires glycine for growth. In the wild type, glycine synthesis is catalyzed by serine hydroxymethyltransferase (EC 2.1.2.1). Cell fractionation by differential centrifugation and isopycnic sucrose gradient analysis reveals that the enzyme activity is found in both the mitochondrial and cytosol fractions. The specific activity in the mitochondrial fraction is about 20 times higher than in the cytosol, and is much more stable to thermal inactivation. The glycine-requiring mutant has lost all of the mitochondrial enzyme activity, while retaining the cytosol activity. The mutant is very stable but can be induced to revert by several chemical mutagens. One glycine-independent revertant induced by ethyl methane sulfonate was studied in detail. Serine hydroxymethyltransferase activity is again present in the mitochondrial fraction, at about $1/3$ of the wild-type level. However, the revertant mitochondrial enzyme exhibits an altered thermal sensitivity, with a half-life at 45° of 55 min as compared to 180 min in the wild type. The half-life for the cytosol enzyme in all three strains is 7 min. Mixing experiments demonstrate that the heat lability of the revertant enzyme is not due to a dissociable factor in the extract. The data are consistent with the idea that the original mutation occurred in the structural gene for one isozyme of the enzyme and that the revertant has undergone a second mutation in this gene, partially restoring enzyme activity.

The heritable change in phenotype exhibited by variant clones of cultured vertebrate cells could be due to two types of events: (1) mutation, i.e., a heritable alteration in the DNA base sequence of a gene; or (2) an epigenetic event, analogous to those presumed to underlie the specialization of cell populations that occurs during development. The second alternative is difficult to test. Mutation, however, is a reasonably well-understood phenomenon, and its occurrence leads to some well-defined predictions. Chemical and physical treatments that are mutagenic in microorganisms should greatly increase the frequency of cell variants; this occurs in several systems (1-3), but not in all (4, 5). The frequency of recessive mutations should decrease markedly with increasing gene dosage; this property is supported in some studies (6) but not in others (5, 7).

A third prediction is that mutation can lead to production of an altered gene product, the base change in the DNA sequence being translated into an amino-acid change in a protein. Evidence has been reported for cell variants that synthesize altered enzymes. The criteria used have been immunological crossreactivity (8), decreased sensitivity to inhibitors (9, 10), and modified chromatographic behavior (9, 10).

We have looked for evidence of an altered gene product in Chinese hamster cells that have been induced to revert from glycine auxotrophy to glycine independence. The glycine requirement in the auxotrophic strain is recessive (11) and is associated with low levels of serine hydroxymethylase (L-serine:tetrahydrofolate 5,10 - hydroxymethyltransferase; SHM; EC 2.1.2.1). This enzyme catalyzes the synthesis of glycine from L-serine:

If the original event that led to the deficiency in enzyme activity were due to a mutation in the structural gene for SHM, then enzyme activity could be restored in a revertant by three mechanisms: (1) a true reversal of the original mutation; (2) a second mutation in the structural gene that leads to a compensatory change in the enzyme structure; and (3) suppression of the original mutation, e.g., by changes in translation. The second mechanism yields an altered protein, and operates in bacteria (12) and in yeast (13).

We describe here the isolation of a revertant of a SHM-deficient mutant of Chinese hamster cell that has regained an altered SHM activity, as indicated by its increased heat lability. Two isozymes of SHM are found in these cells, but only the species located in the mitochondrial fraction is affected by the mutations. The small size of the mitochondrial genome and the recessive nature of the original mutation make it unlikely that a mitochondrial gene is involved.

MATERIALS AND METHODS

Cell Cultures. Clone K1 of a Chinese hamster ovary cell line (1) is considered as the wild type; strain 51-11 is a glycine-requiring subclone of K1 isolated by Kao and Puck (1). Cells were routinely grown in monolayer culture on plastic dishes (Lux) at 37° in an atmosphere of 5% CO_2 . F-12 medium (Gibco) was used (14) supplemented with 10% (v/v) fetal-calf serum (Gibco). When large quantities of cells were needed for preparation of enzyme extracts, the cells were grown in suspension culture under the same conditions, except calcium was omitted from the medium. For selection of glycine-independent colonies, glycine was omitted from the F-12 medium and dialyzed fetal-calf serum was used. Serum was dialyzed by circulating 1 liter of serum through the chamber of a Bio-Fiber 80 Beaker Dialyzer (Bio Rad) at 4° while passing 45 liters of phosphate-buffered saline solution through the dialysis tubing at about 2 liters/hr.

Abbreviation: SHM, serine hydroxymethyltransferase.

Mutagenesis with ethyl methane sulfonate (1) and the selection of glycine-independent revertants have been described (6). After an initial period in glycine-free medium, revertant clones were maintained in complete F-12 medium.

Cell Fractionation. Suspension cultures of 3–4 liters at a density of 4 to 7×10^5 cells per ml were harvested by centrifugation at $600 \times g$ for 5 min at 37° . All subsequent operations were at 0° – 4° . Cell pellets were washed twice with a buffer containing 0.25 M sucrose–10 mM Tris·HCl (pH 7.4)–10 mM NaCl–1 mM EDTA. The cells were resuspended in about 5 ml of the same buffer and broken in a Potter–Elvehjem homogenizer with a motor-driven Teflon pestle. The speed and duration of the homogenization were varied to be just sufficient to break more than 95% of the cells, as judged by microscopic examination. Nuclei and unbroken cells were sedimented by centrifugation at $900 \times g$ for 6 min, and washed with an equal volume of the same buffer. The final viscous pellet was regarded as the nuclear fraction. The combined supernatants were centrifuged again at $900 \times g$ for 5 min, the pellet was discarded, and the supernatant was centrifuged at $10,000 \times g$ for 15 min. This supernatant was again centrifuged at $10,000 \times g$ for 15 min, the final supernatant being the cytosol fraction. The pellet from the first $10,000 \times g$ centrifugation was washed twice with 2 ml of buffer and resuspended in 1–2 ml of buffer. In experiments involving cytochrome oxidase measurements, this mitochondrial fraction was used without further treatment. In all other experiments, the particulate matter was disrupted with 0.1% Triton X-100 for 10 min at 0° , and insoluble material was removed by centrifugation at $12,000 \times g$ for 20 min. The final mitochondrial extract and the cytosol fraction were dialyzed overnight in 2 liters of the same buffer. The protein content of the various fractions was determined by the method of Lowry *et al.* (15).

Enzyme Assays. SHM was measured essentially by the method of Taylor and Weissbach (16). The final reaction mixture contained 0.1 M Tris·HCl (pH 7.4); 5 mM 2-mercaptoethanol; 0.05% Triton X-100; 0.03 mM pyridoxal-5-phosphate; 2.7 mM *dl*,L-tetrahydrofolic acid; 2 mM L-[β - 14 C]serine (Schwartz-Mann) (0.08 Ci/mol); and up to 0.05 ml of extract in a total volume of 0.1 ml. The reaction was initiated by addition of 0.05 ml of a double-strength solution, containing all of the components except the extract, to 0.05 ml of enzyme extract in a 10-ml conical centrifuge tube. After 20 min at 37° the reaction was terminated by addition of 0.25 ml of 0.6 M sodium acetate buffer (pH 4.4) containing 10 mM L-serine and 40 mM formaldehyde as carriers. After the mixture was mixed well, 1.25 ml of 0.4% dimedon solution was added and the solution was heated in a boiling water bath for 20 min to insure a complete reaction between the formaldehyde and dimedon. The precipitated radioactive formaldehyde was extracted with 2 ml of toluene by vigorous Vortex mixing followed by centrifugation in a clinical centrifuge for 1 min. One milliliter of the upper toluene phase was withdrawn and counted in 4.5 ml of toluene-based scintillation fluid.

Cytochrome oxidase was measured by following the decrease in absorbance of a solution of cytochrome *c* at 550 nm in a Zeiss recording spectrophotometer at room temperature. The cuvette contained 40 mM sodium phosphate buffer (pH 6.2), 0.3 mM cytochrome *c*, and up to 0.05 ml of extract in a total volume of 0.5 ml. The cytochrome *c* was reduced just before use by adding a slight molar excess of fresh sodium

ascorbate and then bubbling air through the solution for 15–30 min to destroy the excess ascorbate. Activity was calculated on the basis of a molar extinction coefficient difference of 19,700 between reduced and oxidized cytochrome *c*.

Acid phosphatase was assayed by measuring the hydrolysis of *p*-nitrophenyl phosphate. The reaction mixture contained 0.15 M sodium acetate buffer (pH 5.0) and 5 mM *p*-nitrophenyl phosphate, in a volume of 0.4 ml. Up to 0.02 ml of extract was added to start the reaction. After 30 min of incubation at 37° , 0.1 ml of 6.75 M NaOH was added and the tubes were centrifuged in a clinical centrifuge for 5 min. The absorbance of the supernatant was measured at 410 nm, and the amount of *p*-nitrophenol liberated was calculated with an extinction coefficient of 17,000 (17).

SHM activity was heat-inactivated by heating a cell extract in a capped tube in a water bath. At various times, 0.05-ml aliquots were removed to reaction tubes in ice, and all samples were assayed together at the end of the heating period.

Unless noted otherwise, all chemicals were purchased from Sigma or Fisher.

RESULTS

Reversion of a Glycine Auxotroph. Revertants to glycine independence were sought from populations of strain 51-11, a Chinese hamster cell mutant deficient in SHM activity. When supplemented with glycine, this mutant exhibits a plating efficiency and growth rate similar to the parental wild-type cells (1). In a glycine-free medium, the mutant fails to grow, even if inoculated at cell densities approaching confluency. Spontaneous revertants are extremely rare, occurring at a frequency of less than 10^{-8} . However, revertants can readily be induced by treatment with ethyl methane sulfonate, at an average frequency of 5×10^{-6} glycine-independent colonies per surviving cell.

Colonies that grew in glycine-free medium after mutagenesis of 51-11 were cloned and grown up to large populations. Crude cell extracts (11) were prepared and tested for SHM activity. Extracts from four revertants in which SHM specific activity had increased relative to the mutant were further tested for an altered heat sensitivity of enzyme activity. SHM activity from one revertant clone, strain R13, was significantly more labile at 45° than that from the wild type. This revertant was studied in more detail.

Subcellular Distribution of SHM. Before the properties of SHM in revertant strains were examined further, the distribution of enzyme activity in subcellular fractions in the wild-type strain was determined. In rat and rabbit liver two isozymes of SHM have been reported, one in the mitochondria and one in the cytosol (18, 19). Cells of strain K1 were homogenized and fractionated into a crude nuclear pellet, a "mitochondrial" pellet, and a cytosol supernatant fraction. Cytochrome oxidase activity was monitored as a mitochondrial marker. Table 1 shows that about half of this activity was recovered in the mitochondrial fraction. Most of the remainder was in the nuclear fraction, which also showed considerable contamination with subcellular particles when examined under phase contrast microscopy. This crude nuclear fraction was not purified further and is not considered in most of the discussion to follow. The cytosol fraction contained about 10% of the total cytochrome oxidase activity. However, the specific activity of cytochrome oxidase in the mitochondrial fraction

FIG. 1. Isopycnic sucrose gradient centrifugation of the mitochondrial fraction prepared from strain K1. A 0.1-ml sample of a mitochondrial fraction was layered onto 4.6 ml of a linear sucrose gradient (0.99–1.75 M) in 10 mM Tris·HCl (pH 7.4)–1 mM EDTA. After centrifugation for 145 min at 40,000 rpm at 3° (Spinco SW56 rotor), the bottom of the tube was punctured and approximately 0.2-ml fractions were collected. The bottom of the tube is at the left in the figure. (O) cytochrome oxidase; (Δ) serine hydroxymethylase; (●) acid phosphatase.

was 100 times greater than in the cytosol fraction, indicating that a substantial separation of the mitochondria had been achieved.

Acid phosphatase activity, measured as *p*-nitrophenyl phosphatase, was monitored as a marker for lysosomes (Table 1) (20). The location of this activity predominantly in the cytosol fraction was unexpected, and may partly represent nonlysosomal enzyme activity (21).

More than 40% of the SHM activity in these cells is in fact found in the mitochondrial fraction (Table 1), and the specific activity of the enzyme in this fraction is routinely more than 10 times higher than that found in the cytosol fraction. Treatment of the mitochondrial fraction with 0.1% Triton X-100 or by sonication for 15 sec renders all of the SHM activity nonsedimentable. When the mitochondrial fraction was subjected to isopycnic sucrose density gradient centrifugation (Fig. 1), the cytochrome oxidase and SHM activities banded together at a density of 1.19. Most of the acid phosphatase activity in this fraction also banded at this density. It thus appears that most of the SHM activity in these cells is associated with large particles, probably mitochondria, although other organelles (lysosomes, peroxisomes) cannot be ruled out.

The cytosol SHM activity is too great to represent mitochondrial contamination alone. Heat-inactivation experiments confirm the presence of a second species of enzyme in the cytosol fraction. SHM activity in the mitochondrial fraction is rather stable at 45°, exhibiting an exponential decay with a half-life of about 180 min. In contrast, SHM activity in the cytosol fraction decays with biphasic kinetics; most of the activity has a half-life of about 7 min, while the remainder is quite stable, like the mitochondrial SHM activity (Fig. 2A).

FIG. 2. Heat inactivation of SHM activity from cytosol and mitochondrial fractions at 45°. (A) (●) K1 (wild type) mitochondrial; (○) K1 cytosol; (□) 51-11(gly⁻ mutant) cytosol. (B) (▲) R13 (revertant) mitochondrial; (△) R13 cytosol; (■) R6 (revertant) mitochondrial.

The relative heat resistance of the mitochondrial SHM cannot be due to its localization in particles *per se*, since the enzyme activity was rendered nonsedimentable by treatment with 0.1% Triton X-100 before heat inactivation was done. Detergent treatment similarly has no effect on the heat sensitivity of the cytosol activity.

In summary, two isozymic forms of SHM exist in Chinese hamster cells. One is heat stable and is the only form found in the mitochondrial fraction. It is designated as mitochondrial SHM, but this should be taken to indicate its fractionation behavior rather than as a definite localization in this organelle. The other activity is heat labile and is found in the cytosol fraction along with some mitochondrial SHM. The presence of the latter in the cytosol fraction may be an artifact due to the disruption of some particles during the fractionation.

The distribution of SHM activity in the glycine-requiring mutant strain 51-11 was determined next. This strain exhibits about 10% of the overall wild-type specific activity for SHM.

TABLE 1. Enzyme activities in cell fractions from strain K1 (wild type)

Fraction		Serine hydroxymethylase	Cytochrome oxidase	Acid phosphatase
% Total activity				
	Cytosol	24	9	68
	Mitochondrial	42	51	3
	Nuclear	34	39	29
Total activity* (units)		380	10,620	7,090
Relative specific activity†				
	Cytosol	0.49	0.19	1.36
	Mitochondrial	15.7	19.2	1.21
	Nuclear	0.72	0.87	0.59
Overall specific activity		2.16	106.2	40.4

* One unit of activity is the formation of 1 nmol of product per min.

† Setting the overall specific activity, in units/mg of protein, equal to one.

FIG. 3. Heat inactivation of SHM at 58° in the presence of L-serine (2 mM) and pyridoxal-5-phosphate (0.1 mM). Symbols are the same as in the legend of Fig. 2.

This auxotrophic strain has lost all of its mitochondrial SHM activity, but has retained about half of its cytosol enzyme activity relative to the wild type (Table 2). SHM activity in the nuclear fraction is also greatly reduced in this strain, in agreement with the idea that most of the activity in this fraction is due to particulate contamination.

The glycine-independent revertant strain, R13, exhibits about 50% of the overall wild-type specific activity for SHM. In this strain, the enzyme activity has returned to the mitochondrial fraction to a great extent (Table 2). Cytochrome oxidase activity is similar in the mitochondrial fractions from all these strains (data not shown).

Heat Sensitivity of SHM Activity in Mutants. Heat inactivation of the SHM activity in strain 51-11 was done to determine which form of the enzyme was represented by the residual activity in this glycine-requiring mutant. Fig. 2A shows that all of the activity in this strain is of the heat-labile, cytosol type. Unlike the wild-type strain, none of the SHM activity (<5%) found in the cytosol fraction was of the heat-stable, mitochondrial type. Thus the mutation in strain 51-11 has completely eliminated the mitochondrial SHM activity while leaving the cytosol enzyme unaffected.

The heat sensitivity of SHM activity in fractions of the revertant strain R13 is shown in Fig. 2B. The cytosol activity is entirely of the heat-labile type, just as in the original mu-

FIG. 4. Heat inactivation of SHM activity in mixed extracts. A and B represent independent experiments. The extracts were prepared from mitochondrial fractions that had been solubilized by detergent treatment. (●) K1; (○) R13; (◐) one-half of K1 and one-half of R13. The dashed line represents the expected result from the algebraic sum of the two activities. (A) 0.73 unit of K1 and 1.00 unit of R13; (B) 1.25 units of K1 and 1.14 units of R13.

tant. However, the activity that has returned to the mitochondrial fraction exhibits a much greater heat lability (half-life = 45 min) than the mitochondrial activity in the wild type (half-life = 180 min). In other revertants, SHM of normal heat sensitivity is again found in the mitochondrial fraction, such as strain R6 in Fig. 2B. Heat inactivation under another set of conditions underscores the altered heat sensitivity of the R13 mitochondrial SHM activity. L-Serine and pyridoxal-5-phosphate (a substrate and cofactor) can protect the wild-type cytosol and mitochondrial SHM activity from heat inactivation at temperatures up to 58°. The cytosol SHM activity in strain R13 is similarly protected. The revertant mitochondrial SHM activity again shows a markedly increased heat sensitivity compared to the wild type despite the presence of the protective small molecules (Fig. 3). Inactivation does not follow first-order kinetics, due perhaps to interactions between the enzyme and its effectors.

To elucidate further the nature of the heat lability of the revertant SHM activity, mitochondrial extracts from the wild-type and revertant strain R13 were mixed and then subjected to heat inactivation at 45°. Fig. 4 shows that the mixture is inactivated at a rate expected from the algebraic sum of the two components (dashed line). This result rules out the possibility that a dissociable factor in the cell extracts is responsible for imparting an increased heat sensitivity to an otherwise unaltered enzyme in the revertant.

Comparison of Other Properties of SHM. Several other properties of the mitochondrial SHM activity in the wild-type strain and in the revertant were compared. Both have a pH optimum at 7.5–8.0. The Michaelis constant for L-serine, calculated from reciprocal plots, is 0.4–0.6 mM for both mitochondrial enzyme activities, and it is in the same range (0.5–0.9 mM) for cytosol SHM activities in all three strains. Similarly, the revertant and the mutant enzyme activities do not differ from the wild-type counterparts in the amount of tetrahydrofolic acid needed for maximum activity. Exposure to 2 M urea at 0° inactivates the revertant SHM activity at a faster rate than the wild type, but with complex kinetics (data not shown).

TABLE 2. Specific activities* of serine hydroxymethylase in cell fractions from wild-type and mutant strains

Strain	Phenotype	No. of determinations	Fraction		
			Cytosol	Mitochondrial	Nuclear
K1	Wild type (gly ⁺)	8	2.2 ± 0.4	26.7 ± 2.3	6.7 ± 1.0
51-11	gly ⁻ mutant	3	1.1 ± 0.2	<0.15	0.4 ± 0.1
R13	gly ⁺ revertant	10	2.1 ± 0.4	10.9 ± 2.1	3.7 ± 0.7

* Units/mg of protein. One unit is the production of 1 nmol of formaldehyde per min. Standard errors are given.

DISCUSSION

The existence of two compartmentalized SHM activities in cultured Chinese hamster cells agrees with studies of intact rodents (22). The enzymes purified from the cytosol and from mitochondria in both rat liver (18) and rabbit liver (19) have similar kinetic properties, but can be distinguished either electrophoretically or immunologically. Possible functions for SHM in mitochondria could be to supply glycine for heme synthesis (23) or to provide the one-carbon unit for the synthesis of *N*-formylmethionine. The latter is involved in initiation of mitochondrial protein synthesis (24). It would not be expected that a mitochondrial gene codes for SHM, since the enzyme would represent only a very small fraction of all mitochondrial components and the mitochondrial genome is only large enough to code for a small number of proteins (25). Moreover, a recessive glycine requirement would not be expressed in a cell containing one mutant mitochondrion and a large number of the wild-type organelles.

Two kinds of evidence now strongly support the idea that the lack of mitochondrial SHM in strain 51-11 is due to mutation. First, variants lacking enzyme activity are isolated only after treatment with any one of various mutagens, the spontaneous mutant frequency being less than 3% of that found when the most effective mutagen is used (26). Similarly, revertants back to glycine independence are only found after treatment with chemical mutagens. The spontaneous revertant frequency is less than 0.2% of the frequency induced by ethyl methane sulfonate. Second, among four revertants tested that have regained substantial mitochondrial SHM activity, one clearly differs from the wild type. Both at 45° in the absence of ligands and at 58° in the presence of a substrate and cofactor, the mitochondrial SHM activity of strain R13 is more sensitive to heat inactivation than that of either the wild type or the other revertants. Revertants such as this would be expected if the original mutation were in the structural gene for mitochondrial SHM. They would arise by second-site reversion; that is, a second mutation in the structural gene that introduces a second amino-acid change in the enzyme. This second change can partially or fully restore enzyme activity, but the structure of the enzyme is not identical to the original wild type.

There are alternative explanations for the altered SHM activity of strain R13. The increased heat sensitivity could be due to the lack of post-translational modification such as polymerization or addition of a carbohydrate. This possibility predicts that a hybrid cell formed between the revertant and the wild type would produce only wild-type enzyme activity. Another explanation is that there is yet another isozyme for mitochondrial SHM that is not expressed in the wild type or in most revertants but that has been turned on in strain R13. It is difficult to eliminate this possibility, which can always be

invoked to explain the appearance of a new cell property. Ultimately, amino-acid sequence analysis is required to discover the exact nature of the changes induced by mutagen treatment.

Drug-resistant Chinese hamster cell variants can harbor altered forms of hypoxanthine phosphoribosyl transferase (8), RNA polymerase (9), and dihydrofolate reductase (10). The results described here for auxotrophic cells add SHM to this list, and further support the idea that mutation is the basis for cell variation in these systems.

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