Substitution of a Pentalenolactone-Sensitive Glyceraldehyde-
3-Phosphate Dehydrogenase by a Genetically Distinct Resistant
Isoform Accompanies Pentalenolactone Production in Streptomyces arenæ

KAI-UWE FRÖHLICH,1 MICHAEL WIEDMANN,† FRIEDRICH LOTTSPEICH,2
AND DIETER MECKE1

Physiologisch-chemisches Institut der Universität Tübingen, 7400 Tübingen, and Max-Planck-Institut für Biochemie, Am
Klopferspitz, 8033 Martinsried, Federal Republic of Germany

Received 1 May 1989/Accepted 15 September 1989

Pentalenolactone (PL), an antibiotic produced by Streptomyces arenæ, is a potent inhibitor of glyceraldehyde-
3-phosphate dehydrogenase (GAPDH). The producer strain contains different isoforms of GAPDH: a
PL-sensitive enzyme on nonproduction media and a PL-insensitive enzyme on production media. After
induction of PL synthesis, the sensitive GAPDH disappears parallel to the disappearance of its activity,
as shown by Western (immunoblot) hybridization. The two isoenzymes exhibit little immunological cross-
reactivity and differ in size, amino acid composition, and several amino acid residues of their amino termini.
Two different types of plasmids from a S. arenæ genomic library, named pBRPLR1 and pBRPLR2, were
cloned in Escherichia coli by selection for enhanced PL resistance. Both contain a GAPDH structural gene.
Plasmid pBRPLR1 increases E. coli PL tolerance 7-fold, and plasmid pBRPLR2 increases it 30-fold. GAPDH
from pBRPLR1 transforms shows biphasic PL inactivation kinetics. These cells contain PL-sensitive
GAPDH from both E. coli and S. arenæ. GAPDH from pBRPLR2 transforms tolerates higher PL
concentrations than either E. coli or S. arenæ PL-sensitive GAPDH but is less resistant than S. arenæ
PL-insensitive GAPDH. Nondenaturing polyacrylamide electrophoresis showed this GAPDH to be a hybrid of
E. coli and S. arenæ PL-insensitive GAPDH. The hybrid enzyme could be purified to homogeneity. Induction
of the lacZ promoter of pUC subclones of both GAPDH genes had only a small effect on raising the level of
intracellular GAPDH.

The sesquiterpene antibiotic pentalenolactone (PL) selectively and irreversibly inactivates the NAD+-dependent
glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [N-
glyceraldehyde-3-phosphate:NAD+ oxidoreductase (phosphorylating)] (EC 1.2.1.12) of all procaryotic and eucaryotic
organisms tested so far, binding covalently to the enzyme in a stoichiometric reaction (8, 9, 16, 25). It is therefore a
potent inhibitor of growth when glucose is the obligate
carbon source. The producer strain Streptomyces arenæ is resistant to growth inhibition during antibiotic production
only by forming an isoenzyme of GAPDH which is insensitive to PL. On complex media, when no PL production takes
place, the sole GAPDH that can be detected has a sensitivity to PL similar to that of GAPDH of other organisms.
When PL production is induced, the activity of the PL-sensitive isoenzyme decreases, while the activity of the PL-insensitive
GAPDH increases (27).

Both isoenzymes have been purified and characterized. A marked difference in their molecular weights has been
shown. The molecular weight of 37,000 per subunit of the PL-insensitive GAPDH is similar to that of NAD+-depen-
dent GAPDHs of all other organisms investigated so far (15). The PL-sensitive GAPDH shows the distinctly higher molu-
cular weight of 43,000 per subunit. Earlier studies did not clarify whether the two isoenzymes are convertible by partial proteolysis or whether two structural genes code
different proteins. Furthermore, the question of whether other mechanisms of resistance to this antibiotic exist in the
producer strain remained open. In this paper, we show that these enzymes are encoded by two genes with different
regulation and that resistant Escherichia coli transformants express the PL-insensitive isoenzyme of S. arenæ.

MATERIALS AND METHODS

Chemicals and reagents. Anti-rabbit immunoglobulin G
immunoglobulin G (IgG) from guinea pig was obtained from Renner GmbH (Dannstadt, Federal Republic of Germany).
All restriction endonucleases, DNA ligase, alkaline phosphatase from calf intestine, GAPDH from S. cerevisiae and
rabbit muscle, lactate dehydrogenase from rabbit muscle, horseradish peroxidase (enzyme immunosassay grade), glyceraldehyde-3-phosphate-diacetal (cyclohexylammonium salt), and NAD+ were obtained from Boehringer GmbH, Mannheim, Federal Republic of Germany. Glyceraldehyde-3-phosphate was prepared from the diacetal as described by
the manufacturer. Freund adjuvant was obtained from Sigma
Chemical Co. (St. Louis, Mo.). Bacto-Tryptone and yeast extract were obtained from Difco Laboratories (Detroit, Mich.). DEAE-Sephacel was obtained from Pharmacia Fine
Chemicals (Freiburg, Federal Republic of Germany). Bio-
Gel A 0.5 m and Bio-Gel HTP were obtained from Bio-Rad
Laboratories (Richmond, Calif.), and Ultrogel AcA34 was obtained from LKB (Bromma, Sweden). Microdilution plates with 96 wells were obtained from Greiner, Nüttlingen,
Federal Republic of Germany, and nitrocellulose filters

* Corresponding author.
† Present address: Boehringer Mannheim GmbH, Werk Tutzing,
8132 Tutzing, Federal Republic of Germany.

© 1989, American Society for Microbiology

6696
(HAHY, 0.45 μm) were obtained from Millipore (Bedford, Mass.). PL was isolated as chlorohydrin as previously described (27).

**Strains and media.** *S. arenae* TÜ469 (DSM 40 734) was kindly provided by H. Zähner, Institut für Mikrobiologie, Tübingen, Federal Republic of Germany, and *Streptomyces lividans* TK64 (21) was provided by T. Kieser, John Innes Institute, Norwich, United Kingdom. *E. coli* K-12 RR1 (1) was used for all cloning and gene expression experiments except for pUC subcloning in strain BMH71-18 (28). The mutant strain DF240 (CGGCS963) (gap-7 thi-1 relA lac22 λ spoT) was obtained from D. G. Fraenkel, Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, Mass. (18).

*S. arenae* was grown in a 20-liter fermentor as previously described (27). Wickerham medium was used for the isolation of PL-sensitive GAPDH and of DNA, and synthetic medium was used for the isolation of PL-insensitive GAPDH (27).

**E. coli** cells were grown in 100 ml of M9 medium containing 0.4% glucose (minimal medium) or LB medium (complex medium) in 1-liter Erlenmeyer flasks (24). Ampicillin (40 mg/liter) was used for plasmid maintenance, and 60 mg of isopropyl-β-D-thiogalactopyranoside per liter was used for the induction of the lac promoter. Selection for PL resistance was conducted on M9-glucose plates containing 40 mg of ampicillin per liter and 10 μM PL.

**Enzyme isolation and GAPDH assay.** GAPDH isolation buffer consisted of 5 mM EDTA–5 mM 2-mercaptoethanol–10 μM NAD⁺–50 mM Tris hydrochloride, pH 8.0. PL-sensitive and -insensitive GAPDH from *S. arenae* were isolated as described previously (27). The same protocol was followed for the isolation of GAPDH from *E. coli* wild type and transformants. For small amounts of cell extract, *E. coli* cells were harvested in late exponential growth, washed with GAPDH isolation buffer, sonicated three times for 30 s each, and centrifuged for 10 min at 18,000 × g. The supernatant was used as a cell extract.

Protein was determined by the Bradford method (2). GAPDH activity was measured as described in reference 27. PL was tested by GAPDH inactivation as described in reference 27.

**Protein analysis.** For the amino acid composition analysis, 10 μg of lyophilized GAPDH was hydrolyzed in 50 μl of 6 M HCl–0.05% phenol for 1 h at 155°C. The amino acids were derivatized with α-phthalaldehyde-3-mercaptopropionic acid, separated by high-performance liquid chromatography, and detected by fluorescence spectroscopy as described in reference 14.

**Amino-terminal sequence analysis was performed in a gas-phase sequencer (model 470A) from Applied Biosystems, and the phenythiohydantoin amino acids were identified by high-performance liquid chromatography as described in reference 23. For density gradient centrifugation, a linear sucrose gradient was cast from 2.6 g of 30% sucrose and 2.6 g of 10% sucrose in GAPDH isolation buffer. The sample was applied in a volume of 50 μl. The gradient was collected in fractions of three drops after centrifugation in a SW65 Ti swing-out rotor (Beckman Instruments, Inc., Palo Alto, Calif.) at 180,000 × g and 4°C for 16 h. The molecular weight was calculated as described in reference 26 by using rabbit muscle lactate dehydrogenase (molecular weight, 144,000) as the standard.

**Immunological techniques.** For antibody production, rabbits were injected subcutaneously with 200 μg of purified GAPDH in complete Freund adjuvant emulsion and boosted after 3 and 6 weeks with 100 μg of GAPDH in incomplete Freund adjuvant. Bleeding was started 2 weeks after injection 3. The immunoglobulins were precipitated from the serum with 50% saturated ammonium sulfate and redissolved in Tris-buffered saline (0.9% NaCl–20 mM Tris hydrochloride, pH 7.4).

Horseradish peroxidase (HRP) was coupled to the immunoglobulins by the periodate method of Nakane and Kawai (30) as described in reference 33. The complex was purified by chromatography on Ultrogel AcA 34.

For sandwich enzyme-linked immunosorbent assay (ELISA), the buffers, incubation, and staining methods were as described in reference 12. Anti-GAPDH antisera were diluted 1:10⁻³ for coating. Extracts containing GAPDH were incubated at 37°C for 3 h, and the HRP-coupled anti-GAPDH antibody (diluted 1:50) was incubated for 2 h. A₄₁₀ was measured with an ELISA reader (Dynatech Industries, Inc., McLean, Va.).

Antibody titers were determined by coating each 300-μl well with 100 μl of 0.001% GAPDH overnight and incubating it with antisera of different dilutions for 3 h at 37°C. The wells were incubated with HRP-coupled anti-rabbit-immunoglobulin antibodies diluted 1:50 after being washed and then processed as described above.

The protocol described in reference 4 was used for Western (immunoblot) transfer and hybridization after sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The anti-GAPDH antibody was diluted 1:200 for the first incubation, and HRP-coupled anti-rabbit-immunoglobulin antibodies were diluted 1:100 for the second incubation. The staining was then conducted with 0.05% 4-chloro-1-napthol, 0.009% H₂O₂, and 17% methanol in Tris-buffered saline.

**Molecular biology.** A genomic library of *S. arenae* total DNA was established by partial digestion with the restriction endonuclease Sau3AI, integration in the BamHI site of the *E. coli* plasmid pBR322, and introduction into *E. coli* K-12 RR1 competent cells.

Restriction digests and ligations were conducted as described by the supplier (Boehringer GmbH). *S. arenae* total DNA was isolated as described in references 6 and 19. A 35-μg portion of the DNA was digested incompletely with 0.9 U of Sau3AI for 1 h to produce 4- to 15-kilobase-pair (kbp) fragments. The fragments were ligated with 1 μg of pBR322 that had been cut with *Bam*HI and dephosphorylated with alkaline phosphatase from calf intestine. *E. coli* was transformed by the calcium chloride procedure as described in reference 24. Plasmids were isolated as described in reference 13.

The protocol described in reference 36 was used for coupled in vitro transcription-translation of plasmid-encoded genes in the extracts of *Streptomyces* cells. A 2-mg portion of plasmid was incubated with 8 μl of synthesis mix and 2 E₃₀₀ units of *S. lividans* TK64 extract.

**Electrophoresis.** The Laemmli system (22) was used for sodium dodecyl sulfate-polyacrylamide gel electrophoresis with 10% acrylamide separation gels. Nondenaturing electrophoresis was performed as described in reference 7.

Molecular weight determination by electrophoresis on nondenaturing gels (11, 17) was conducted with a series of 5, 6, 7, 8, and 9% acrylamide gels.

Acrylamide gels were stained with silver (32) for visualization of total protein. GAPDH was detected in nondenaturing gels by equilibration with 50 mM sodium pyrophosphate–5 mM EDTA–20 mM sodium arsenate–10 μM NAD⁺, pH 8.5 for 30 min at 4°C, and incubation in 50 mM sodium pyrophosphate–5 mM EDTA–20 mM sodium arsenate–10 μM NAD⁺, pH 8.5 for 1 h at 37°C.
TABLE 1. Amino acid composition of GAPDHs from different organisms

| Amino acid* | S. arenæ | Bacillus steatorrhophilus (37) | Thermus aquaticus (20) | E. coli (3) | Lobster (5) | Bee (5) |
|-------------|----------|--------------------------------|------------------------|------------|-------------|---------|
|             | PL-sensitive | PL-insensitive | PL-sensitive | PL-insensitive | PL-sensitive | PL-insensitive | PL-sensitive | PL-insensitive | PL-sensitive | PL-insensitive | PL-sensitive | PL-insensitive | PL-sensitive | PL-insensitive | PL-sensitive | PL-insensitive | PL-sensitive |
| Ax | 37 | 40 | 41 | 34 | 43 | 43 | 32 | 36 | 32 | 43 | 43 | 32 | 36 | 32 | 43 | 43 | 32 | 36 |
| Thr | 14 | 26 or 27 | 18 | 22 | 27 | 20 | 18 | 2 |
| Ser | 21 | 15 | 17 | 15 | 15 | 25 | 19 | 2 |
| Glx | 26 | 31 | 26 | 23 | 20 | 24 | 24 | 2 |
| Gly | 38 | 37 | 38 | 24 | 30 | 30 | 30 | 2 |
| Ala | 33 | 35 | 38 | 42 | 35 | 32 | 37 | 2 |
| Val | 29 | 21 | 43 | 30 | 34 | 38 | 32 | 2 |
| Met | 4 | 6 | 7 | 7 | 10 | 5 | 2 |
| Ile | 22 | 18 | 19 | 22 | 20 | 18 | 24 | 2 |
| Leu | 34 | 31 or 32 | 26 | 31 | 20 | 18 | 20 | 2 |
| Tyr | 7 | 8 | 8 | 10 | 8 | 9 | 12 | 2 |
| Phe | 9 | 12 | 5 | 7 | 11 | 15 | 12 | 2 |
| His | 10 | 7 | 9 | 10 | 6 | 5 | 6 | 2 |
| Lys | 18 | 21 | 23 | 22 | 27 | 28 | 30 | 2 |
| Arg | 18 | 9 | 15 | 16 | 12 | 16 | 9 | 2 |

* Proline, tryptophan, and cysteine were not determined.

0.012% nitrotetrazolium blue–0.002% phenazinemethosulfate–3 mM NAD+–0.4 mM glyceraldehyde-3-phosphate for 1 to 2 h in the dark at room temperature (modified from reference 10). The stained gels were stored in 7% acetic acid.

RESULTS

Structural relationship of GAPDH isoenzymes. Both PL-sensitive and PL-insensitive GAPDH isoenzymes were purified from S. arenæ as described in Materials and Methods, and their amino acid compositions were compared (Table 1). Despite the high similarity of both isoenzymes with the composition of GAPDHs from other organisms, several distinct differences were obvious between PL-sensitive and PL-insensitive GAPDH. While the PL-sensitive enzyme contains 26 or 27 threonine and 9 arginine residues, the PL-insensitive enzyme contains 14 threonine and 18 arginine residues.

The first 20 residues of the amino-terminal amino acid sequences (Fig. 1), show a high homology between both S. arenæ isoenzymes with other GAPDHs. However, the differences in residues 1, 18, and 20 of the S. arenæ isoenzymes, as well as the differences in the amino acid composition (e.g., arginine and threonine), contradict the assumption of an interconversion by limited proteolysis.

Antibody production and cross-reactivity. Purified preparations of both isoenzymes from S. arenæ were used as antigens to immunize rabbits. In Western hybridization studies, antiserum against PL-sensitive GAPDH reacted with PL-sensitive GAPDH from S. arenæ and with yeast GAPDH. No hybridization could be detected with PL-insensitive GAPDH from S. arenæ and E. coli GAPDH. Anti-PL-insensitive-GAPDH serum reacted strongly with PL-insensitive GAPDH from S. arenæ and with yeast GAPDH and weakly with PL-sensitive GAPDH from S. arenæ and E. coli GAPDH. Both antisera showed no reaction with rabbit GAPDH. Some cross-reactivity was observed in ELISA between PL-sensitive and PL-insensitive GAPDH from S. arenæ.

Degradation of PL-inactivated GAPDH. The activity of PL-sensitive GAPDH and the immunological response to anti-PL-sensitive GAPDH antiserum disappeared simultaneously in cells of S. arenæ which had been transferred from complex media to minimal medium (Fig. 3). The enzyme seems to be degraded proteolytically immediately after inactivation by the PL. The rapid disappearance rate of the PL-sensitive GAPDH protein following loss of enzymatic activity is in accordance with the observation that, in vitro, PL inactivation of GAPDH renders the enzyme proteolytically labile. While 30 µg of native rabbit muscle GAPDH in 1 ml of 50 mM Tris hydrochloride–5 mM 2-mercaptoethanol–1 mM NAD+, pH 8.0 remained essentially unaffected for at least 100 h at 29°C in the presence of 0.65 µg of clostristain, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, GAPDH was totally hydrolyzed within 7 h after PL inactivation.

Cloning of S. arenæ GAPDH genes in E. coli. A genomic library consisting of 20,000 independent clones with an average insert size of 5 kb was produced from 15 µg of S. arenæ total DNA. Transformants tolerating a higher concentration of PL than wild-type E. coli were selected by plating of the cells on minimal medium containing 10 µM PL. Eleven transformants grew with selection for PL resistance. Ten of these contained the same type of plasmid, named pBRPLR1, with a 4.0-kb insert of Streptomyces DNA, and one transformant contained the plasmid pBRPLR2 with a...
3.1-kbp insert. Both plasmids conferred the phenotype of elevated PL resistance on retransformation. They were mapped with 14 different restriction enzymes. No similarities or overlaps of the inserts could be detected (Fig. 4).

**Characterization of the E. coli transformants.** While untransformed E. coli and a control transformed with the vector pBR322 were inhibited in growth by 9 μM, the MIC was 60 μM PL for transformants of pBRPLR1 and 300 μM PL for those of pBRPLR2. The GAPDH level in the crude extracts of both transformants was 30 to 50% lower than that of RR1 transformed with the vector pBR322 or of untransformed cells.

[FIG. 3. Inactivation kinetics of PL-sensitive GAPDH in S. arenae after PL formation. At time zero, the cells were transferred from nonproduction to production medium. PL (+) was determined in culture supernatant. PL-insensitive GAPDH (△) was measured in cell extracts as the residual activity after incubation with 4 μM PL for 1 h, and PL-sensitive GAPDH (■) was estimated as the difference of total and PL-insensitive GAPDH activity. Antigenic response to anti-PL-sensitive GAPDH antibody (□) was measured by ELISA and given as a percentage of the initial value.]

To determine whether the two plasmids isolated carry the structural genes for the Streptomyces plasmid, the plasmids were introduced into the E. coli mutant strain DF240, which carries a temperature-sensitive GAPDH. The mutant is unable to grow on glucose as the sole carbon source at 42°C and only grows poorly on glucose at 30°C. After transformation with either pBRPLR1 or pBRPLR2, the cells grew like the wild type at 30°C but still failed to grow at 42°C. Crude extracts of the transformants exhibited a two- to fourfold increase of GAPDH activity compared with the untransformed mutant or DF240 transformed with plasmid pBR322.

The isolated plasmids were used in a coupled, in vitro transcription-translation system from S. lividans. A total of 270 pkat of GAPDH was produced from 2 μg of purified pBRPLR1-DNA, and 130 pkat was produced from 2 μg of pBRPLR2. Formation of GAPDH was not observed when the assay mixture was incubated without a plasmid or with pBR322. Significant amounts of newly synthesized GAPDH could be detected by ELISA in the in vitro assays containing pBRPLR1 and pBRPLR2.

In cell extracts, GAPDH from pBRPLR2 clones was inactivated by lower concentrations of PL than was PL-insensitive GAPDH from S. arenae but was still 10 times more resistant than the PL-sensitive GAPDH from S. arenae and 100 times more resistant than E. coli GAPDH. In crude extracts of pBRPLR1 transformants, 80% of the GAPDH activity could be inactivated with low PL levels, comparable with untransformed E. coli GAPDH. The residual 20% of GAPDH activity was 10 times more PL resistant, behaving like S. arenae PL-sensitive GAPDH (Fig. 5).

After separation of cell extracts by polyacrylamide gel electrophoresis and staining for GAPDH activity, extracts from pBRPLR1 clones showed two bands of activity, one exhibiting the mobility of E. coli GAPDH and the other exhibiting the mobility of S. arenae PL-sensitive GAPDH (Fig. 6). In extracts of pBRPLR2 clones, at least two bands were visible, migrating between the bands of E. coli GAPDH and S. arenae PL-insensitive GAPDH.

All GAPDH activity of E. coli transformants carrying plasmid pBRPLR2 failed to bind to a DEAE-cellulose col-

[FIG. 5. GAPDH inactivation of cell extracts of E. coli RR1 transformants carrying plasmids pBR322 (○), pBRPLR1 (△), and pBRPLR2 (△) and of purified S. arenae PL-sensitive (□) and PL-insensitive (■) GAPDH. GAPDH (670 pkat) was incubated with different concentrations of PL for 1 h at room temperature. Glycerol-phosphate was added, and the residual activity was measured. The 100% value is the activity without PL addition.]

Downloaded from http://jb.asm.org/ on July 24, 2018 by guest
um in GAPDH isolation buffer, while E. coli GAPDH and both PL-sensitive and -insensitive GAPDH from S. arenae did bind to a DEAE-cellulose column. The molecular weight of the purified enzyme was estimated by sucrose gradient centrifugation and non-denaturing electrophoresis by the method of Ferguson (11) (Table 2). The results from the Ferguson plot (11, 17) showed that the charge of the isolated GAPDH from E. coli carrying pBRPLR2 has an intermediate value compared with those of GAPDH from untransformed E. coli and of PL-insensitive GAPDH from S. arenae.

Subcloning the GAPDH genes from pBRPLR1 and pBRPLR2. Various restriction fragments from pBRPLR1 and pBRPLR2 were subcloned into the vectors pUC18 and pUC19 and tested for their abilities to transform E. coli K-12 BMH7118 to PL resistance. The 1.8-kbp EcoRI fragment of pBRPLR1 (plasmids pUCPLR1-1 and pUCPLR1-19) and the 2.3-kbp XhoI-BamHI fragment of pBRPLR2 (plasmids pUCPLR1-2 and pUCPLR1-19) sufficed for expression of PL resistance. On induction with isopropyl-β-D-thiogalactopyranoside the level of GAPDH expressed in the transformants depended on the orientation of the Streptomyces DNA fragment in the pUC vector. One orientation (pUCPLR1-1 and pUCPLR1-19; orientation as marked by arrows in Fig. 4) led to a doubled level of GAPDH on induction for both inserts, while in the opposite orientation (pUCPLR1-1 and pUCPLR1-19) GAPDH levels were unaffected by isopropyl-β-D-thiogalactopyranoside.

### Table 2. Molecular weight of native GAPDHs

| GAPDH type          | Molecular weight as determined by: | Sucrose density gradient centrifugation | Native polyacrylamide gel electrophoresis (Ferguson plot) |
|---------------------|------------------------------------|----------------------------------------|----------------------------------------------------------|
| S. arenae (PL sensitive) | 179,000                           | 179,000                                |                                                          |
| S. arenae (PL insensitive) | 149,500                           | 144,000                                |                                                          |
| E. coli (untransformed) | 140,000                           | 144,000                                |                                                          |
| E. coli (pBRPLR1) (larger form) | 179,000                           | 179,000                                |                                                          |
| E. coli (pBRPLR1) (smaller form) | 140,000                           | 144,000                                |                                                          |
| E. coli (pBRPLR2)     | 151,000                           | 146,000                                |                                                          |

### DISCUSSION

Both GAPDH isoenzymes of S. arenae were investigated for their immunological cross-reactivity to establish whether they are encoded by the same gene, with the PL-insensitive form being released by a limited proteolysis of the sensitive form, or whether the two enzymes are encoded by different genes which are regulated conversely. While antisera against both enzymes strongly bound to yeast GAPDH, little to no cross-reactivity could be detected between the two enzymes from S. arenae, which makes a common origin from the same gene unlikely. The amino acid compositions of both S. arenae GAPDHs show similarity to one another and to GAPDHs of other organisms. Major differences were observed in the arginine and threonine content. PL-sensitive GAPDH contains 26 or 27 threonine and 9 arginine residues, while PL-insensitive GAPDH contains 14 threonine and 18 arginine residues. This also contradicts the idea of a conversion of PL-sensitive GAPDH to PL-insensitive GAPDH. The amino termini of all GAPDHs show a striking homology in the area of amino acids 1 to 15. The higher molecular weight of the S. arenae PL-sensitive GAPDH is, therefore, not caused by an amino-terminal elongation. Nevertheless, differences in 5 of the first 19 amino acid residues prove that the two S. arenae GAPDH isozymes must be encoded by different genes.

The PL-sensitive GAPDH from S. arenae, though more than 1,000-fold less resistant to PL compared with S. arenae PL-insensitive GAPDH, still tolerates a 70-times-higher PL concentration than E. coli GAPDH. This low-level resistance may enable the cell to produce the energy necessary for the isozyme switching even when a low PL level exists from surrounding S. arenae cells or during the first phase of PL production. The higher tolerance of both S. arenae GAPDH enzymes to PL compared with the E. coli enzyme was used for selection to isolate the two GAPDH genes from S. arenae. The two types of plasmid isolated gave rise to different levels of PL resistance. A PL-degrading activity could not be found in extracts of the transformants (results not shown). In vitro expression of the genes localized on the plasmids and the complementation of temperature-sensitive GAPDH mutants of E. coli proved that both plasmids carry a GAPDH structural gene. The immunological data led to the conclusion that pBRPLR1 codes for the PL-sensitive GAPDH and that pBRPLR2 codes for the PL-insensitive GAPDH from S. arenae. This is confirmed by the improvement of growth of the E. coli GAPDH mutant at 30°C after transformation. The failure of the transformant to grow at 42°C cannot be explained. It may be caused by a faster degradation of the foreign protein, which is further enhanced by the elevated temperature, leading to a steady-state level of functional GAPDH in the cell which is too low to allow growth on glucose.

Subclones of both GAPDH genes confined the regions available for the location of the open reading frames and defined the direction of transcription. For both genes, the induction of gene expression, when controlled by the lacZ promoter, gave rise to a doubled level of enzyme. This is a rather weak effect for induced expression on a high-copy-number plasmid. When cloned on pBR322, the presence of either of the S. arenae GAPDH genes even lowers the total GAPDH contents of E. coli wild-type cells. The presence of the foreign GAPDH genes seems to have a repressing effect on the expression of the native GAPDH gene, and additionally, both S. arenae GAPDHs seem to be degraded rather rapidly in E. coli.
A fundamental difference between the two clones was revealed by activity staining after electrophoresis. In transformants of plasmid pBRPLR1, the PL-sensitive GAPDHs from S. arenae and E. coli were both detected. They seem to coexist independently in the cell. In transformants of pBRPLR2, neither S. arenae PL-insensitive GAPDH nor E. coli GAPDH were found. Instead, two or three forms of GAPDH were stained, showing an electrophoretic mobility intermediate of the two enzymes expected. Mixed GAPDH tetramers consisting of subunits from different organisms have been formed by mixing enzymes from different sources (34). Some enzymes seem to be more disposed to form mixed tetramers than others. The lack of tendency of S. arenae PL-sensitive GAPDH to form hybrids may be due to its unusual subunit size. It can be speculated that this size difference prevents the formation of mixed GAPDH tetramers in S. arenae, which would be degraded after a partial inactivation by PL, leading to the loss of part of the newly synthesized PL-insensitive GAPDH subunits.

The formation of hybrids in clones of pBRPLR2 and the absence of hybrids in clones of pBRPLR1 explain the results of PL inactivation experiments in cell extracts. The E. coli-S. arenae PL-insensitive GAPDH hybrids show logarithmic inactivation kinetics, like that of a uniform GAPDH with an intermediate PL sensitivity compared with S. arenae PL-insensitive GAPDH and E. coli GAPDH. The two GAPDH isoenzymes in pBRPLR1 clone extracts are inactivated independently, resulting in biphasic kinetics.

Similar switching of homologous proteins, as observed in S. arenae, from a sensitive to a resistant form on antibiotic production has been described for a ribosomal component in Streptomyces kanamyceticus (31) and for DNA gyrase subunit B in Streptomyces sphaeroides (35). When expressed in S. lividans, the gyrB gene from S. sphaeroides leads to the formation of highly novobiocin-resistant gyrase from two S. lividans gyrase A subunits and two S. sphaeroides gyrase B subunits and a gyrase of intermediate resistance, presumably containing one subunit B from S. lividans and one from S. sphaeroides. This parallels our finding of the formation of a GAPDH hybrid tetramer with an intermediate PL resistance in E. coli containing the S. arenae PL-insensitive GAPDH gene.

The development of a complicated regulatory system involving two enzymes for the same function and degradation of the antibiotic-sensitive form with antibiotic production, instead of simply the use of the resistant protein throughout, should be of some advantage for the cell. In the case of S. arenae, the PL-insensitive GAPDH has a 3.4-times-lower maximal activity than the PL-sensitive enzyme (550 nkat/mg compared with 1,870 nkat/mg of pure enzyme [27]). This reduction of enzyme efficiency may be a side effect of the higher resistance. It may be economically advantageous to produce four times less of this major protein during most of the life cycle.

ACKNOWLEDGMENTS

We thank Harold Taylor for critical reading of the manuscript, Andrea Braithwaite for technical assistance, and E. Hannapel for help with the amino acid analysis.

This work was supported by the Deutsche Forschungsgemeinschaft and the Fonds der Chemischen Industrie.

LITERATURE CITED

1. Bolivar, F., R. L. Rodriguez, P. J. Greene, M. C. Betlach, H. L. Heynker, and H. W. Boyer. 1977. Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. Gene 2:95–113.
2. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72:248–254.
3. Branten, G., and C. Branten. 1985. Nucleotide sequence of the Escherichia coli gap gene. Eur. J. Biochem. 150:61–66.
4. Burnett, W. N. 1981. “Western blotting”: electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radiolabeled protein A. Anal. Biochem. 112:195–203.
5. Carlson, C. W., and R. W. Brosemer. 1971. Comparative structural properties of insect triose phosphate dehydrogenases. Biochemistry 10:2113–2119.
6. Chater, K. F., D. A. Hopwood, T. Kieser, and C. J. Thompson. 1982. Gene cloning in Streptomyces. Curr. Top. Microbiol. Immunol. 96:69–95.
7. Davis, B. J. 1964. Disc electrophoresis. II. Method and application to human serum proteins. Ann. N.Y. Acad. Sci. 121:404–427.
8. Duszenko, M., H. Balla, and D. Mecke. 1982. Specific inactivation of glucose metabolism from eucaryotic cells by pentalenolactone. Biochim. Biophys. Acta 714:344–350.
9. Duszenko, M., and D. Mecke. 1986. Inhibition of glyceraldehyde-3-phosphate dehydrogenase by pentalenolactone in Trypanosoma brucei. Mol. Biochem. Parasitol. 19:223–229.
10. Entian, K.-D., K.-U. Fröhlich, and D. Mecke. 1984. Regulation of enzymes and isoenzymes of carbohydrate metabolism in the yeast Saccharomyces cerevisiae. Biochim. Biophys. Acta 799:181–186.
11. Ferguson, K. A. 1964. Starch-gel electrophoresis—application to the classification of pituitary proteins and polypeptides. Metabolism 13:985–1002.
12. Franke, M., S. Rohrschneider, and R. Geiger. 1982. Enzyme immunoassay of human urinary kallikrein. J. Clin. Chem. Clin. Biochem. 20:621–626.
13. Fröhlich, K.-U., K.-D. Entian, and D. Mecke. 1984. Cloning and restriction analysis of the hexokinase II gene of the yeast Saccharomyces cerevisiae. Mol. Gen. Genet. 194:144–148.
14. Graser, T. A., H. G. Godel, S. Albers, P. Földi, and P. Fürst. 1985. An ultra rapid and sensitive high-performance liquid chromatographic method for determination of tissue and plasma free amino acids. Anal. Biochem. 151:142–152.
15. Harris, J. I., and M. Waters. 1976. Glyceraldehyde-3-phosphate dehydrogenase, p. 1–49. In P. D. Boyer (ed.), The enzymes, vol. 13. Academic Press, Inc., N.Y.
16. Hartmann, S., J. Neeff, U. Heer, and D. Mecke. 1978. Areanaemycin (pentalenolactone): a specific inhibitor of glycolysis. FEBS Lett. 93:339–342.
17. Hedrick, J. L., and A. J. Smith. 1968. Size and charge isomer separation and estimation of molecular weights of proteins by disc gel electrophoresis. Arch. Biochem. Biophys. 126:155–164.
18. Hillman, J. D., and D. G. Fraenkel. 1975. Glyceraldehyde 3-phosphate dehydrogenase mutants of Escherichia coli. J. Bacteriol. 122:1175–1179.
19. Hintermann, G., R. Cramerl, T. Kieser, and R. Hätter. 1981. Restriction analysis of the Streptomyces glaucescens genome by agarose gel electrophoresis. Arch. Microbiol. 130:218–222.
20. Hocking, J. D., and J. I. Harris. 1980. D-Glyceraldehyde 3-phosphate dehydrogenase. Eur. J. Biochem. 108:567–579.
21. Hopwood, D. A., T. Kieser, H. M. Wright, and M. J. Bibb. 1983. Plasmids, recombination and chromosome mapping in Streptomyces lividans 66. J. Gen. Microbiol. 129:2257–2269.
22. Laemmlli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) 227:680–685.
23. Loitspeich, F. 1985. Microscale isocratic separation of phenylthiohydantoin amino acid derivatives. J. Chromatogr. 326:321–327.
24. Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
25. Mann, K., and D. Mecke. 1979. Inhibition of spinach glyceral-
dehydroy-3-phosphate dehydrogenase by pentalenolactone. Nature (London) 282:535–536.

26. Martin, R. G., and B. N. Ames. 1961. A method for determining the sedimentation behavior of enzymes: application to protein mixtures. J. Biol. Chem. 236:1372–1379.

27. Maurer, K.-H., F. Pfeiffer, H. Zehnder, and D. Mecke. 1983. Characterization of two glyceraldehyde-3-phosphate dehydrogenase isoenzymes from the pentalenolactone producer Streptomyces arenae. J. Bacteriol. 153:930–936.

28. Messing, J., B. Gronenborn, B. Müller-Hill, and P. H. Hofschneider. 1977. Filamentous coliphage M13 as a cloning vehicle: insertion of a HindIII fragment of the lac regulatory region in M13 replicative form in vitro. Proc. Natl. Acad. Sci. USA 74:3642–3646.

29. Misset, O., J. van Beeumen, A.-M. Lambeir, R. van der Meer, and F. R. Oppenhoe. 1987. Glyceraldehyde-phosphate dehydrogenase from Trypanosoma brucei. Eur. J. Biochem. 162:501–507.

30. Nakane, P. K., and A. Kawaoi. 1974. Peroxidase-labelled antibody: a new method of conjugation. J. Histochem. Cytochem. 22:1084–1091.

31. Nakano, M. M., H. Mashiko, and H. Ogawara. 1984. Cloning of the kanamycin resistance gene from a kanamycin-producing Streptomyces species. J. Bacteriol. 157:79–83.

32. Oakley, B. R., D. R. Kirsch, and N. R. Morris. 1980. A simplified ultrasensitive silver stain for detecting proteins in polyacrylamide gels. Anal. Biochem. 105:361–363.

33. O’Sullivan, M. J., and V. Marks. 1981. Methods for the preparation of enzyme-antibody conjugates for use in enzyme immunoassay. Methods Enzymol. 73:147–166.

34. Suzuki, K., and J. I. Harris. 1975. Hybridization of glyceraldehyde-3-phosphate dehydrogenase. J. Biochem. 77:587–593.

35. Thiara, A. S., and E. Cundiff. 1988. Cloning and characterization of a DNA gyrase B gene from Streptomyces sphaeroides that confers resistance to novobiocin. EMBO J. 7:2255–2259.

36. Thompson, J., S. Rae, and E. Cundiff. 1984. Coupled transcription-translation in extracts of Streptomyces lividans. Mol. Gen. Genet. 195:39–43.

37. Walker, J. E., A. F. Carne, M. J. Runswick, J. Bridgen, and J. I. Harris. 1980. d-Glyceraldehyde-3-phosphate dehydrogenase. Eur. J. Biochem. 108:549–565.