The Aes Protein and the Monomeric α-Galactosidase from *Escherichia coli* Form a Non-covalent Complex

IMPLICATIONS FOR THE REGULATION OF CARBOHYDRATE METABOLISM*

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Aes, a 36-kDa acetylesterase from *Escherichia coli*, belongs to the hormone-sensitive lipase family, and it is involved in the regulation of MalT, the transcriptional activator of the maltose regulon. The activity of MalT is depressed through a direct protein-protein interaction with Aes. Although the effect is clear-cut, the meaning of this interaction and the conditions that trigger it still remain elusive. To perform a comparative thermodynamic study between the mesophilic Aes protein and two homologous thermostable enzymes, Aes was overexpressed in *E. coli* and purified. At the last step of the purification procedure the enzyme was eluted from a Mono Q HR 5/5 column as a major form migrating, anomalously, at 56 kDa on a calibrated Superdex 75 column. A minor peak that contains the Aes protein and a polypeptide of 50 kDa was also detected. By a combined analysis of size-exclusion chromatography and surface-enhanced laser desorption ionization-time of flight mass spectrometry, it was possible to demonstrate the presence in this peak of a stable 87-kDa complex, containing the Aes protein itself and the 50-kDa polypeptide in a 1:1 ratio. The homodimeric molecular species of Aes and of the 50-kDa polypeptide were also detected. The esterase activity associated with the 87-kDa complex, when assayed with *p*-nitrophenyl butanoate as substrate, proved 6-fold higher than the activity of the major Aes form of 56 kDa. Amino-terminal sequencing highlighted that the 50-kDa partner of Aes in the complex was the α-galactosidase from *E. coli*. The *E. coli* cells harboring plasmid pT7-SCII-*aes* and, therefore, expressing Aes were hampered in their growth on a minimal medium containing raffinose as a sole carbon source. Because α-galactosidase is involved in the metabolism of raffinose, the above findings suggest a potential role of Aes in the regulation of carbohydrate metabolism in *E. coli*.

The *Escherichia coli aes* (or *ybac*) gene encodes the 36-kDa cytoplasmic protein Aes (1), a carboxylesterase belonging to the hormone-sensitive lipase family (HSL)[1] (2). Although the physiological role of this enzyme is still obscure, it has recently received greater attention due to the discovery that Aes is involved in the regulation of the maltose regulon in *E. coli* (reviewed in Ref. 3). The *E. coli* maltose system consists of 10 genes encoding proteins for the uptake and metabolism of maltodextrin and maltose (3). MalT is the transcriptional activator of the maltose regulon and the prototype of a new family of transcription factors (4, 5) and integrates several signals through its amino-terminal three domains (6). To activate *mal* genes transcription MalT acts together with the cyclic AMP-catabolite protein complex, the inducer maltotriose, and ATP (7). Several regulatory circuits modulate the activity of MalT, *e.g.* MalT expression is regulated by Mlc, a glucose-inducible repressor (8). In addition, MalT is negatively controlled by at least three proteins: MalY, MalK, and Aes. MalY is an enzyme with cystathionine β-lyase activity (9, 10), which interacts directly with the amino terminus of MalT (11, 12), seemingly through a patch made up of a hydrophobic core surrounded by highly polar residues (13). MalK, the ATP-hydrolyzing subunit of the maltose transport system (14), has been reported to interact directly with MalT (15), probably through some residues localized in its C-terminal domain (16). With regard to the Aes protein, it has been demonstrated that Aes controls MalT activity through a direct protein-protein interaction that counteracts the binding of the MalT effector maltotriose, and it was postulated that the Aes binding site is mostly located in DT1 domain of MalT, which contains also the ATP binding site (17). Accordingly, ATP and ADP differently affect the competition between Aes and the inducer (maltotriose) as regards the binding to MalT. A working model is one in which maltotriose stabilizes the ATP-bound form of MalT that consequently is more prone to oligomerization to bind DNA, whereas Aes and/or MalY antagonise(s) this effect and in doing so it (they) prevent(s) oligomerization. The one or more sites of interaction on MalT of Aes and MalY are probably superimposed, whereas MalK would appear to interact at a different location (12). It is possible that MalY and Aes share similar structural determinants, but these cannot be easily predicted on the basis of sequences analysis only (13).

It has been reported that the basal level of *aes* expression is very low (1). However, the introduction of a plasmid harboring the *aes* gene was required to allow *E. coli* growth on minimal medium supplemented with triacetylglycerol (triacetin) as sole carbon source. Moreover, the existence of a repressor of the *aes* gene has also been postulated (1), thus suggesting the importance of this enzyme in particular growth conditions.

We have been working for several years on the structure-function relationship in thermostable carboxylesterases from the HSL family (18, 19). Recently, we reported the three-dimensional structures of EST2 from *Alicyclobacillus acidocaldarius*, a thermophilic eubacterium (20), and of the homologous

[1] The abbreviations used are: HSL, hormone-sensitive lipase; *p*NP, *p*-nitrophenyl; LB, Luria-Bertani; IPTG, isopropyl-β-D-thiogalactoside; SELDI-TOF, surface enhanced laser desorption ionization-time of flight; MS, mass spectrometry; EST2, esterase 2; AFEST, Archaeoglobus fulgidus esterase.

enzyme AFEST, from the hyper-thermophilic archaeon *Archaeoglobus fulgidus* (21). We also reported some structural and thermodynamic studies on both enzymes (22–24). To perform a comparative analysis with a mesophilic member of the HSL family we started the cloning of *aes* from *E. coli* genomic DNA, the overexpression of the protein in *E. coli*, and its purification. Quite surprisingly we isolated a complex, which had never been previously described, of the Aes enzyme with an *E. coli* 50-kDa polypeptide that we demonstrate here to be the α-galactosidase, an enzyme belonging to the family 4 of the glycosylhydrolases. This enzyme is implicated in the degradation of α-galactosides such as galactose oligosaccharides, galactomannans, and galactolipids (25), and therefore directly involved in the *E. coli* carbohydrate metabolism.

EXPERIMENTAL PROCEDURES

Chemicals—*p*-Nitrophenyl (*p*NP) butanoate, *p*NP-α-D-galactopyranoside, *p*NP-α-D-arabinopyranoside, Fast Blue RR, β-naphthyl acetate, and raffinose were purchased from Sigma Chemical Co. (St. Louis, MO).

Strains and Plasmids—*E. coli* DH5α strain (Invitrogen, Carlsbad, CA) was used as the host for cloning, whereas *E. coli* BL21(DE3) cells harbored the recombinant plasmid for gene expression. The *aes* gene was amplified from *E. coli* genomic DNA by polymerase chain reaction with the Expand high fidelity PCR system (Roche Molecular Biochemicals), using oligonucleotides *ybacoli-nde* (5′-GGAGTTTTCATAT-GAAGCCGGAAAACAAACTA-3′) and *ybacoli-pst* (5′-TCTACAGTC-GACCTGCAGATCGAAATGATGGCTA-3′) as forward and reverse primers, respectively. The amplification reaction was performed in a 30-cycle PCR (1 min at 94 °C, 1 min at 55 °C, and 1 min at 72 °C). The amplification primers *ybacoli-nde* and *ybacoli-pst* were designed to introduce *Nde*I and *Pst*I restriction sites (underlined) at the 5′ and 3′ ends of the gene, respectively. The amplified band was cut with restriction enzymes *Nde*I and *Pst*I, purified with an agarose gel-extraction kit (Quiaex II, Quiagen), and ligated into the *Nde*I/*Pst*I-linearized expression vector pT7-SCII (26) to create a pT7-SCII-*aes* construct. The cloned fragment was completely sequenced to verify that no undesired mutations were introduced during amplification.

Overexpression and Purification—The *aes* gene was expressed under the direct control of isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible promoter of the φ*10* gene (26). *E. coli* BL21(DE3) cells were transformed with the pT7-SCII-*aes* construct and cultured at a large scale in 5 liters of Luria-Bertani (LB) medium supplemented with 100 μg/ml ampicillin. Cells, at a density corresponding to an optical density value of 1 at 600 nm, were treated with 0.5 mM IPTG for 4 h. Thereafter, cells (40 g wet weight) were harvested by centrifugation (6,000 × *g*, 4 °C, 10 min), washed with 25 mM Tris-HCl buffer (pH 8.5)/2.5 mM MgCl₂/0.5 mM EDTA (buffer A), and stored at −20 °C. Finally, when required, cells were thawed and re-dissolved in 100 ml of buffer A. Disruption was achieved with a French Press cell, and cellular debris were removed by centrifugation (30,000 × *g*, 4 °C, 20 min).

Purification of the recombinant enzyme was adapted from a previously described procedure for EST2 and AFEST (18, 19). The crude extract was treated with 10% (w/v) ammonium sulfate and centrifuged (30,000 × *g*, 4 °C, 20 min). The supernatant was made 40% (w/v) ammonium sulfate and centrifuged again (30,000 × *g*, 4 °C, 20 min). The pellet was re-dissolved in 100 ml of buffer A and loaded onto a Q-Sepharose Fast Flow column (20 × 2.6 cm, Amersham Biosciences, Uppsala, Sweden) equilibrated in buffer A. After 2 volumes of washing, a linear gradient of NaCl (from 0 to 1 M) was applied. Active fractions were pooled, made 10% (w/v) in ammonium sulfate, and loaded onto a HiLoad 16/10 Phenyl-Sepharose column (Amersham Biosciences, Uppsala, Sweden) equilibrated in buffer A containing 10% ammonium sulfate. Proteins were eluted by a decreasing gradient (10% to 0%) of ammonium sulfate in buffer A. Esterase activity was eluted at the end of the gradient. Active fractions were pooled, dialyzed against buffer A, and fractionated by a linear gradient of NaCl (0–1 M) onto a Mono Q HR 5/5 column run over an FPLC apparatus (Amersham Biosciences, Uppsala, Sweden). Most of the applied activity was eluted at 300 mM NaCl in a large peak (peak A), whereas a small peak (peak B) was also observed at 380 mM NaCl. The most active fractions were pooled and stored at 4 °C, until used.

Enzyme Assays—The time course of the esterase-catalyzed hydrolysis of *p*NP butanoate, *p*NP-α-D-galactopyranoside, and *p*NP-α-D-arabinopyranoside was followed by monitoring of *p*-nitrophenoxide production at 405 nm, in a 1-cm path-length cell with a CARY 100 Scan spectrophotometer (Varian, Victoria, Australia). Initial rates were calculated by linear least-squares analysis of time courses comprising less than 10% of the total turnover.

Esterase assays were performed at 25 °C in mixture of 40 mM Na₂HPO₄/NaH₂PO₄/4% acetonitrile (pH 7.1) containing *p*NP butanoate at different concentrations. Stock solutions of *p*NP butanoate were prepared by dissolving the substrate in pure acetonitrile.

α-Galactosidase assays were performed at 25 °C in a mixture of 40 mM Na₂HPO₄/NaH₂PO₄/1 mM MgCl₂/1 mM NADH (pH 7.1) containing 1.5 mM *p*NP-α-D-galactopyranoside or *p*NP-α-D-arabinopyranoside. One unit of enzymatic activity was defined as the amount of protein releasing 1 μmol of *p*-nitrophenoxide/min from *p*NP butanoate or *p*NP-galactoside at 25 °C. The absorption coefficient used for *p*-nitrophenoxide was 17,000 M⁻¹ cm⁻¹. The protein concentration was measured with the Bio-Rad Protein Assay system, using bovine serum albumin (Fraction V) as standard.

Kinetic Measurements—Initial velocity *versus* substrate concentration data were fitted to the Lineweaver-Burk transformation of the Michaelis-Menten equation, by weighted linear least-squares analysis with a personal computer and the Grafit program (27). Assays were done in duplicate or triplicate, and results for kinetic data were the mean of two independent experiments. Substrate concentration was in the range 10–1000 μM.

Densitometry Analysis—The ratio of Aes and α-galactosidase, in samples containing both proteins, was calculated by densitometric analysis of gels scanned by using the image acquisition device ChemiDoc (Bio-Rad), equipped with the software Quantity One, according to the manufacturer's specifications. To calculate the specific esterase activity of Aes alone, the absolute amount of Aes in the complex was calculated on the basis of a Aes calibration curve obtained by running different amounts of purified Aes onto the same gel.

Size-exclusion Chromatography—Size-exclusion chromatography experiments were performed with a High Load 16/60 Superdex 75 column run over an FPLC apparatus. The column was equilibrated and eluted with buffer A containing, when indicated, 500 mM urea. The flow rate was 0.2 ml/min. The column was calibrated in the condition outlined above, using the following molecular mass markers (Amersham Biosciences, Uppsala, Sweden): cytochrome *c* (12,500 Da), ovalbumin (43,000 Da), bovine serum albumin (67,000 Da), and blue dextran (2000 kDa).

Electrophoresis—Electrophoretic runs were performed with a Bio-Rad Mini-Protean II cell unit, at room temperature. SDS-PAGE was performed essentially as described by Laemmli (28). Gels were stained with Coomassie Brilliant Blue G-250. Molecular mass markers (Prestained SDS-PAGE Standard Broad Range, Bio-Rad) were: myosin (205 kDa), β-galactosidase (120 kDa), bovine serum albumin (84 kDa), ovalbumin (52 kDa), carbonic anhydrase (36 kDa), soybean trypsin inhibitor (30 kDa), lysozyme (22 kDa), and aprotinin (7.4 kDa).

15% non-denaturing PAGE at alkaline pH was performed using the same buffer and loading solutions of SDS-PAGE lacking SDS and β-mercaptoethanol. As to the *in situ* esterase activity staining, after electrophoretic run, gels were washed in 25 mM Tris-HCl buffer (pH 8.5)/2.5 mM MgCl₂/0.5 mM EDTA for 2 h and then were incubated in a solution (100 ml) of 100 mM Tris-HCl (pH 7.5) containing 5 mg of β-naphthyl acetate and 25 mg of Fast Blue RR at room temperature. After 5 min of incubation, the reaction was stopped by rinsing with tap water.

Automated Edman Degradation—A sample from the Superdex 75 column (fraction number 23) containing the 87-kDa complex (see "Results") was subjected to 10% SDS-PAGE to separate the 36- and 50-kDa bands. Thereafter, bands were electrotransferred onto Immobilon-P membranes, stained with Coomassie Blue as above, and then submitted for amino-terminal sequence analysis. Only the band corresponding to an apparent molecular weight of 50,000 was excised and subjected to amino acid sequence analysis on an ABI Procise protein sequencer (29), according to the manufacturer's pulse-phase protocol. The determined amino acid sequence was then used to search against the European Bioinformatics Institute protein data base using the Fasta 33 algorithm (30).

SELDI-TOF Mass Spectrometry Analysis—Surface-enhanced laser desorption ionization-time of flight mass spectrometry (SELDI-TOF) analysis was carried out using the Ciphergen Protein Chip System (Ciphergen, Palo Alto, CA). SELDI analysis was performed using a hydrophobic H4 protein chips, containing a long chain aliphatic surface. The samples were applied to a spot, washed with 5% acetonitrile, and allowed to dry. To the dry spot 0.5 μl of matrix was added and allowed to crystallize. The matrix was a saturated solution of sinapinic acid
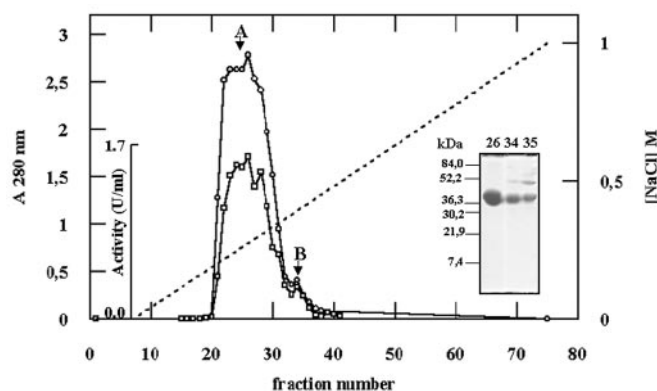
FIG. 1. **Last purification step of recombinant Aes on Mono Q HR 5/5.** Protein was followed by absorbance at 280 nm (○) or by activity (□) with substrate *p*NP butanoate. The main esterase peak (*peak A*) and the small one (*peak B*) were analyzed by SDS-PAGE (*inset*). For clarity, only the fractions indicated by numbers are shown.

(Sigma Chemical Co., St. Louis, MO), 50% acetonitrile, and 0.1% trifluoroacetic acid. The protein chips were transferred to the Ciphergen SELDI reader and analyzed by Ciphergen SELDI Protein Biology System II externally calibrated with myoglobin, equine (16,951.5 Da); horseradish peroxidase (43,240 Da); and Conalbumin, chicken (77,490 Da). The mass identification was performed by averaging at least 50 laser shots (laser intensity = 290) of various regions of the ProteinChip surface.

Aes and α-Galactosidase Activity in Cell Cultures Grown on Chemically Defined Medium Containing Raffinose as Sole Carbon Source—E. coli BL21(DE3) cells transformed with vector pT7-SCII, or with or without the *aes* gene, were grown in LB medium containing ampicillin (100 μg/ml) and cultured at a cell density corresponding to an optical density value of 0.2 at 600 nm. The cultures were 20-fold diluted in minimal medium M9 (50 ml) containing 2% raffinose as carbon source and ampicillin (100 μg/ml). A second culture of *E. coli* transformed with pT7-SCII-*aes* was prepared as above but supplemented with 0.5 mM IPTG. After 4, 20, and 28 h of incubation, an aliquot (15 ml) of each culture was harvested by centrifugation (3000 × *g*, 4 °C, 5 min), cells were re-dissolved in 5 ml of 25 mM Tris-HCl buffer (pH 8.5) and disrupted with a French Press cell. Cellular extracts were recovered by centrifugation. Portions of 100 and 10 μl of each extract were used for the α-galactosidase and esterase assays, respectively.

RESULTS

Cloning, Overexpression, and Purification of Aes Protein— The gene *aes* (also known as *ybac*) from *E. coli* encoding the esterase Aes (2) was cloned from *E. coli* genomic DNA by PCR amplification. Two oligonucleotides (see "Experimental Procedures") were designed to introduce the restriction sites *Nde*I and *Pst*I at the 5′ and 3′ ends of the gene, respectively. The PCR product (of the expected size) was purified from an agarose gel, cut with *Nde*I and *Pst*I, and ligated into the expression vector PT7-SCII, which had been digested with the same restriction enzymes. DNA sequencing confirmed that no undesired mutations were introduced into the gene. The cloning procedure adopted put the gene under the control of the IPTG-inducible $\phi10$ promoter (26), and the protein was expressed without tags being added at the amino- or carboxyl-terminal sides. The overexpression in BL21(DE3) cells was obtained by 4-h induction with 0.5 mM IPTG, and the enzyme was purified as described under "Experimental Procedures." At the final purification step, proteins were fractionated by a linear gradient of NaCl (0–1 M) onto a Mono Q HR 5/5 column using an FPLC apparatus (Amersham Biosciences, Uppsala, Sweden). Most of the applied activity was eluted in a large peak (peak A) at 300 mM NaCl, whereas a small peak (peak B) was also observed at 380 mM NaCl (Fig. 1). The SDS-PAGE analysis (Fig. 1, *inset*) showed that the Aes protein, with an apparent molecular weight of 36,000, was present alone in peak A (frac-
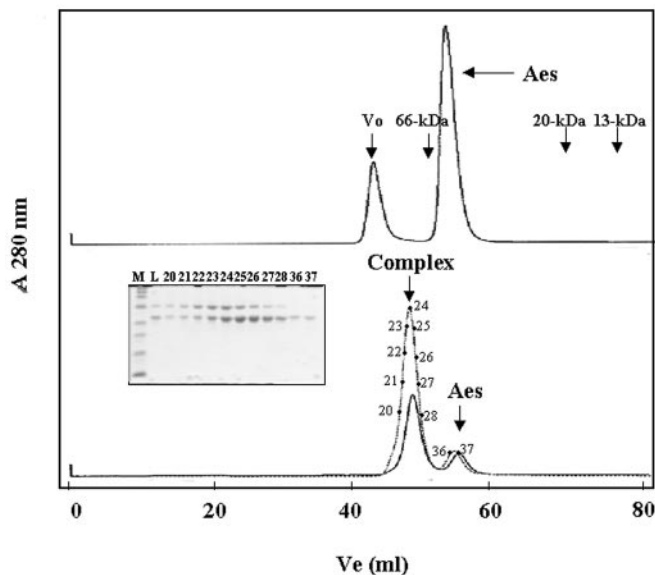

FIG. 2. **Gel filtration analysis on high load Superdex 75.** *Upper trace*: Peak A from Mono Q HR 5/5 loaded together with blue dextran (*Vo*). The elution of markers used for the column calibration is shown by *arrows*. *Lower traces*: Peak B filtrated in the absence (*dotted trace*) or presence (*continuous trace*) of 500 mM urea. *Inset*: SDS-PAGE analysis of fractions from column developed in the absence of urea. Fractions analyzed are indicated by *numbers* on the *graph*. Prestained molecular weight markers (*M*) are: myosin (205 kDa), β-galactosidase (120 kDa), bovine serum albumin (84 kDa), ovalbumin (52.2 kDa), carbonic anhydrase (36.3 kDa), soybean trypsin inhibitor (30.2 kDa), lysozyme (21.9 kDa), and aprotinin (7.4 kDa). *L* represents an aliquot of the material loaded onto the column.

tion number 26) and displayed a specific activity of 28 units/mg, when assayed with *p*NP butanoate as substrate at 25 °C. Peak B (*inset* of Fig. 1; fractions 34 and 35) contained a second band of an apparent molecular weight of 50,000 in addition to the 36-kDa band. Quite interestingly, the esterase specific activity of Aes alone (103 units/mg) in peak B (measured as described under "Experimental Procedures") appeared higher (about 4-fold) as compared with that present in peak A.

*Identification of a 87-kDa Complex Containing Aes and a 50-kDa Polypeptide—*To explain the difference in specific activities said above, having suspected a physical interaction between Aes and the 50-kDa polypeptide, the proteins from both peaks A and B were analyzed by gel filtration experiments. An aliquot (1 ml, 4.5 mg of protein) from peak A was loaded onto a HiLoad 16/60 Superdex 75 column (Amersham Biosciences, Uppsala, Sweden), previously calibrated as described under "Experimental Procedures." It gave a single peak of apparently 56 kDa (Fig. 2, *upper trace*), which was a value higher than expected on the basis of the Aes sequence (about 36,000) and in agreement with the result of 57 kDa reported by Joly *et al.* (17). These authors suggested that Aes is either an asymmetric monomer or a dimeric form delayed in its elution because of interactions with the column matrix. The possibility of a dynamic equilibrium between the monomer and a dimer was ruled out, because there was no dependence from protein concentration (17).

When an aliquot of peak B (1 ml, 5 mg of protein obtained from the Mono Q column) was similarly analyzed by gel filtration, it was found to consist of a major peak of apparently 89 kDa and a minor one eluting at the same position where the 56-kDa Aes form was eluted (Fig. 2, *lower dashed trace*). The SDS-PAGE analysis (*inset* of Fig. 2) showed that the 89-kDa peak (fractions 20–28) contained again the 50- and 36-kDa bands and that the 56-kDa peak contained the 36-kDa band (fractions 36 and 37).
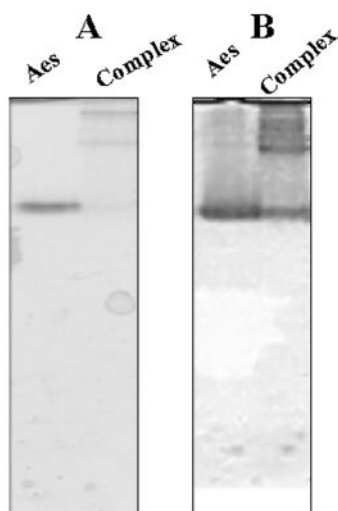
Aes and α-Galactosidase Interaction

FIG. 3. **15% non-denaturing electrophoresis of Aes or complex.** An aliquot of Aes or of complex from the gel-filtration column was fractionated onto a 15% non-denaturing PAGE and stained by Coomassie Brilliant Blue G (*A*) or by *in situ* activity (*B*) as described under "Experimental Procedures."

A careful analysis by densitometry of the SDS-PAGE bands corresponding to the fractions from the major peak was performed. It indicated that the ratio between the intensity of 36- and 50-kDa bands ranged from 1:1 in fractions 20–24, to 2:1 in fractions 25 and 26, to 3:1 in fraction 27. To explain this elution pattern, we hypothesized that the Aes protein could be involved in a putative complex with a 50-kDa form (theoretical molecular weight 86,700) and that this complex co-migrated with a dimeric form of Aes. The Aes protein found in the minor peak of Superdex 75 could derive either from a cross-contamination with the monomeric Aes in the previous step or from a slow dissociation of dimeric Aes. The 50-kDa polypeptide cannot be MalT, which interacts directly with Aes (17), given the large difference in their respective molecular masses (50 *versus* 96 kDa) (4, 5).

In an attempt to clarify the relationship between Aes and the 50-kDa polypeptide in the 89-kDa peak, we performed a 15% non-denaturing PAGE followed by either Coomassie Blue staining (Fig. 3*A*) or by *in situ* activity staining (*panel B*). As shown in the figure most of the protein (and activity) associated with monomeric Aes (*Aes lanes*) migrated at a very different position with respect to the one or more complexes (*Complex lane*), which yielded slowly migrating bands, as well as one minor band migrating at the same position of monomeric Aes, all displaying esterase activity.

A two-dimensional gel analysis (data not shown) proved inconclusive given that the 50-kDa polypeptide in part was associated with the first band shown in Fig. 3*A* (*Complex lane*) and in part was spread along all the gel, particularly in association with the remaining active bands, suggesting a dissociation of the complex during the electrophoresis run.

To assess whether the monomeric Aes observed both in the gel filtration and the non-denaturing PAGE was generated by the 89-kDa complex, we re-chromatographed the major peak (89 kDa) from previous gel-filtration on the same column equilibrated in buffer A and containing 500 mM urea. The rational of this experiment was to determine which protein-protein interactions, within the one or more complexes, were destroyed by the presence of urea. As shown in Fig. 2 (*lower continuous trace*), the complexes-containing peak eluted at the same position at which it was eluted in the absence of urea; in addition, a small peak was eluted at a position slightly delayed with

respect to the monomeric Aes (*dotted trace*). Finally, the ratio between the two peaks appeared reduced, indicating a partial conversion from the higher to the lower form. The SDS-PAGE (data not shown) showed the Aes protein and the 50-kDa polypeptide in a 1:1 ratio in the major peak, the 36-kDa Aes band in the smallest peak, and a tiny amount of the 50-kDa form between both. In conclusion, it is likely that the Aes protein forms a stable 1:1 complex with a polypeptide of 50 kDa, which co-migrates with a dimeric Aes and that the latter dissociates in 500 mM urea. The small increase in the band of 50 kDa between the complex and Aes may be ascribed to a minor dissociation either of the complex or of a putative minor 50-kDa dimeric form or of both. The urea seems also to affect the migration of the monomeric Aes, because it eluted at a slightly lower molecular weight (about 53 kDa), compared with the run without urea. This result suggests a conformational switch in the monomeric Aes that can be responsible for its anomalous migration on the Superdex 75 column. In fact, when the same sample, after a prolonged incubation with 500 mM urea (1 month at 4 °C) and urea removal by diafiltration, was run over a Superdex 200 analytical column, it gave an active Aes migrating at a molecular weight closer to the expected 36,000 value, whereas the non-incubated Aes migrated again with a molecular weight of 56,000 (data not shown).

SELDI-TOF Mass Spectrometry Analysis and Identification of the 50-kDa Polypeptide as the E. coli α-Galactosidase—To investigate the molecular masses of the species mentioned above, an aliquot of fraction 23 obtained by gel filtration and consisting of the 89-kDa peak (Aes:50 kDa ratio of 1:1, Fig. 2, *lower dotted trace*) was examined by SELDI-TOF mass spectrometry. As shown in Fig. 4*A*, the fraction 23, from the Superdex 75 column (Fig. 2), contained two major molecular species, exhibiting mass values of 36,038 and 50,665.7 Da corresponding to Aes and 50-kDa polypeptide. We also observed species with higher masses (Fig. 4*B*) consistent with the theoretical masses of the Aes homodimer (72,078.3 Da), Aes/50-kDa complex (86,703.7 Da), and of 50-kDa homodimer (101,332.5 Da), previously hypothesized on the basis of size-exclusion chromatography. The preincubation of sample with urea at 500 mM before the analysis confirmed that the Aes/50-kDa complex was stable to this treatment (data not shown).

To identify the 50-kDa polypeptide, Aes and 50-kDa proteins were separated by 10% SDS-PAGE, under reducing conditions. The separated proteins were electro-transferred onto Immobilon-P membrane, and the band corresponding to the 50-kDa polypeptide was subjected to amino-terminal sequencing analysis.

The sequence obtained was MMTAPKITFIGAGSTIFV, which by a Fasta 33 analysis (30) was found to correspond to the amino-terminal of the *E. coli* α-galactosidase (Swiss-Prot code: P06720). Our sequence has a threonine in the third position, whereas the sequences available in the Swiss-Prot and TrEMBL and GenBank™ databases have a serine. Because we are quite confident that our sequence is correct, we have concluded that the difference could be strain-specific. In the corresponding sequence from *Salmonella typhimurium* (Q8Z1P0), there is in fact a threonine in that position.

Enzymatic Activities Associated with the 87-kDa Complex—As mentioned before, a considerable difference was noted when comparing the esterase activity related to the Aes band from Peak B with the activity of the pure Aes from Peak A. The Aes protein in the complex was measured as described under "Experimental Procedures." In Fig. 4 the esterase activity, measured by using the same amounts of Aes (about 1 μg), either alone or in the Aes/α-galactosidase complex, was compared by means of Lineweaver and Burk analysis. Surpris-

FIG. 4. **SELDI-TOF mass spectrometry analysis.** Species from the *left* to the *right* of the spectrum, *panel A*: double-charged monomer $(M+2H)^+$, monomer $(M+H)^+$, and double charged $(2M+2H)^+$ dimeric species and $(2M+H)^+$ dimers. In the *inset*, *panel B*, enlargement of the peak corresponding to the dimeric species.
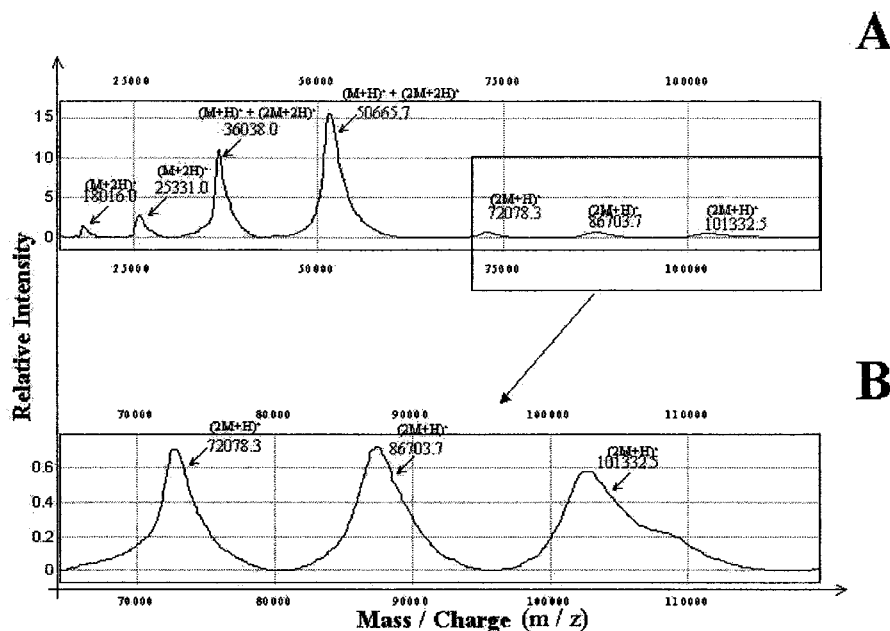
ingly, the Aes activity in the fraction obtained from the gel filtration column and containing mostly the complex (Aes/α-galactosidase ratio 1:1; fraction 24) proved ~6-fold higher in terms of V_{max} than in the monomeric form (Fig. 5 and Table I), thereby confirming the result obtained with the Mono Q column analysis. The K_m for substrate *p*NP butanoate instead was unaffected, as demonstrated by convergence of the two straight lines in Fig. 6 to the *x*-axis yielding the same value of 800 μM, which is in good agreement with a previously reported value (2). As shown in Table I, this activation effect is specific for the Aes/α-galactosidase interaction and can only be partly ascribed to the presence of dimeric Aes for the following reasons: (i) in the fraction containing the heterodimer and dimeric Aes (Aes/α-galactosidase ratio 2:1; fraction 25 from the Superdex 75 column) the activation effect was significantly lower (2.5-fold activation); (ii) with the same fraction the activation was significantly reduced (to 1.4-fold) in the presence of 500 mM urea or when the enzyme was preincubated for 5 min with 500 mM urea before the assay (a treatment that seems to disrupt the Aes dimer; see Fig. 2); (iii) the 6-fold activation in fraction 24, which contains only the complex, was unaffected by 500 mM urea. Urea had no observable effect on the monomeric Aes. Phenylmethylsulfonyl fluoride (1.2 mM), a specific inhibitor of serine-type esterase, inhibited esterase activity in both the Aes alone and in the complex. AgCl (0.05 mM), one of the reported inhibitors of α-galactosidases, also inhibited Aes in all fractions. By using classic substrates for α-galactosidases such as *p*NP-α-D-glucopyranoside and *p*NP-α-D-galactopyranoside, no activity was detected with monomeric Aes, either with fractions enriched in dimeric Aes or in the complex.

Physiological Meaning of the Aes/α-Galactosidase Interaction—Having demonstrated an *in vitro* physical interaction between Aes and α-galactosidase, the next problem was to establish a physiological meaning, if any, for such phenomenon. Because Aes has already been reported to be involved in the negative regulation of MalT, the activator of *mal* genes, its similar interaction with, and likely negative effect on α-galactosidase, prompted us to speculate on a potential and more general role of Aes in the regulation of carbohydrate metabolism. To test this hypothesis we set up an experiment to demonstrate the *in vivo* interaction between Aes and α-galactosidase. We allowed *E. coli* cells, which had been transformed with pT7-SCII-*aes* or pT7-SCII (vector alone), to grow in a chemi-
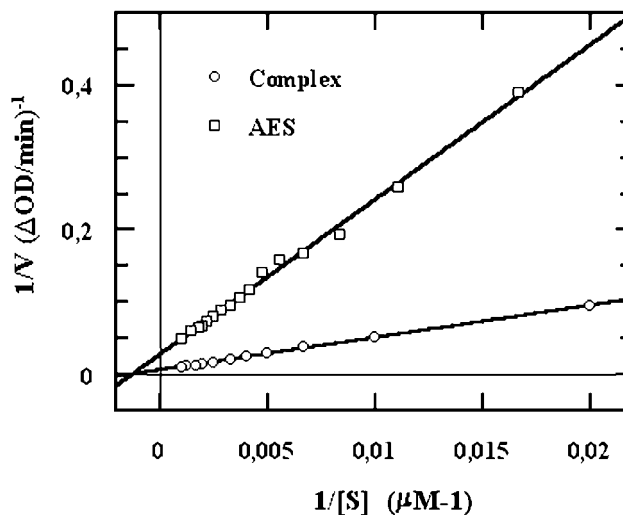

FIG. 5. **Lineweaver and Burk plot of activity associated with Aes or complex.**

cally defined medium supplemented with 2% raffinose as unique carbon source, with or without 0.5 mM IPTG. As shown in Fig. 6, cell growth was slightly reduced without 0.5 mM IPTG but was consistently delayed when Aes was overexpressed by IPTG induction. We also analyzed the time course of the total esterase and α-galactosidase activity (as units/mg of protein) in the cultures. In crude extracts of cells transformed with pT7-SCII, we found low total esterase and high α-galactosidase activity. In contrast, in the culture of cells transformed with pT7-SCII-*aes*, which were not induced with IPTG, the α-galactosidase activity was decreased (by about half), and the total esterase activity raised to high levels. Lastly, in IPTG-induced cells, we found that α-galactosidase activity was undetectable, and the same, or reduced, level of total esterase activity was found compared with the non-induced culture. We concluded that the Aes protein is expressed at a certain level even in the absence of IPTG, as already observed for the thermophilic homologs EST2 and AFEST[2] and that the amount of Aes protein produced is able to partially inhibit α-galactosidase activ-

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[2] L. Mandrich, M. Rossi, and G. Manco, unpublished observations.

TABLE I
Effect of different compounds on esterase activity of Aes or Aes/Agal fractions

Esterase activity was performed in standard assay (40 mM phosphate buffer, 4% acetonitrile, 1 mM *p*NP butanoate at 25 °C). Fractions 24 and 25 were from the gel filtration depicted in Fig. 2 (*lower dotted curve*).

	Esterase activity				
	Without additions	PMSF[a] (1.2 mM)	AgCl[a] (0.05 mM)	Urea (500 mM)	Urea[a] (500 mM)
			m units/mg		
Aes	28	0.3	15	33	28
Aes/Agal 1:1 (fr. 24)	170	0.2	21	180	155
Aes/Agal 2:1 (fr. 25)	80	0.1	8	40	38

[a] 5-min incubation before the assay. Data are mean of two assays, and the S.E. was about 10%.

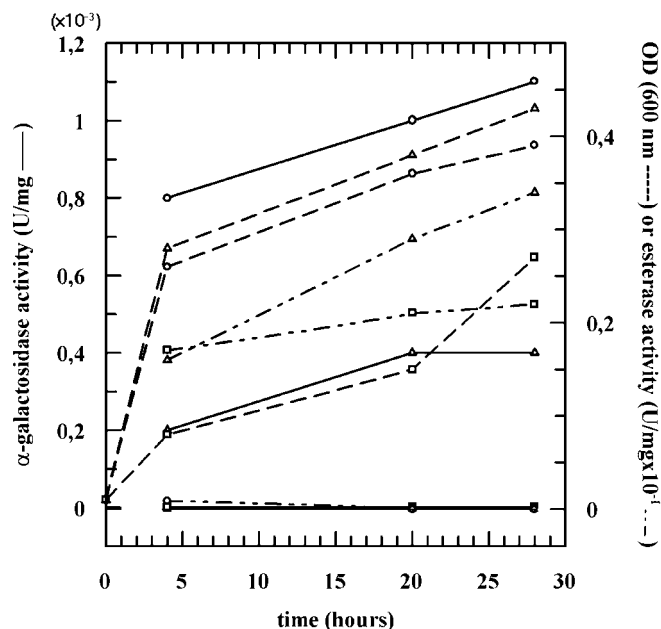

FIG. 6. **Esterase and α-galactosidase activity in *E. coli* cells grown on minimal medium containing 2% raffinose as carbon source.** Shown are the cells absorbance (*dashed lines*), the general esterase activity (*solid lines*), and the α-galactosidase activity (*dashed* and *dotted lines*) at 4, 20, and 28 h for cells containing plasmid pT7-SCII alone (○), pT7-SCII-aes (△), or pT7-SCII-aes IPTG-induced (□), respectively.

FIG. 7. **In vitro effect of purified Aes protein on the α-galactosidase activity.** The *in vitro* effect of purified Aes protein on the α-galactosidase activity was assessed by adding the indicated amounts of pure Aes to crude extracts from cells grown on minimal medium supplemented with 2% raffinose and harboring plasmid pT7-SCII. After 20-min incubation at room temperature α-galactosidase activity was assayed as reported under "Experimental Procedures."

ity. Induction of Aes protein during growth on raffinose was able to depress *E. coli* growth, and this effect was accompanied by disappearance of α-galactosidase activity. In conclusion, Aes depressed α-galactosidase activity *in vivo*.

To ascertain whether this effect also takes place *in vitro* when α-galactosidase is fully active, we added different amounts of pure Aes to the same amount (100 μl) of crude extract of the non-induced culture, containing 1.3×10^{-3} units/mg of α-galactosidase activity. After 20-min incubation at room temperature, activity was assayed. As shown in Fig. 7, α-galactosidase activity decreased proportionally to the amount of Aes protein added.

DISCUSSION

Aes Can Form Complexes with Itself and α-Galactosidase— The biochemical analysis reported here demonstrates the existence of stable Aes complexes with either itself (homodimers of 72 kDa) or with the *E. coli* α-galactosidase (a 87-kDa heterocomplex). This has been demonstrated by a combined analysis of gel-permeation chromatographies, non-denaturing PAGE, and SELDI-TOF MS. We confirmed the data previously reported by Joly *et al.* (17) of an anomalous migration of the Aes recombinant protein (56 kDa) on Superdex columns. It is likely that a particular conformation of the monomeric Aes and not a dimer delayed in its elution, because of interactions with the
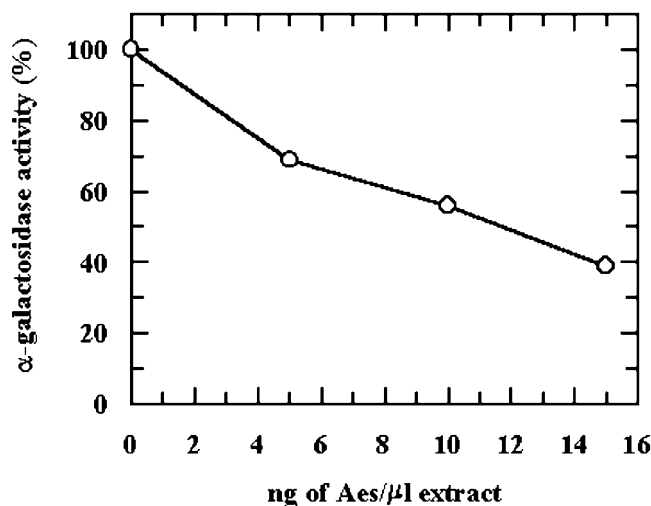
matrix, can be responsible for this behavior. In fact, after prolonged incubation in 500 mM urea and urea removal by diafiltration, the Aes protein was eluted from a Superdex 200 analytical column at a molecular weight closer to the expected value of 36,000 (data not shown). Because the enzyme is still active after this treatment, we excluded the possibility that it was a denatured form of the enzyme. Moreover, a dimeric Aes specie of 72 kDa was actually detected by SELDI-TOF MS and separated from the monomeric Aes by gel filtration. A conformational exchange in Aes protomer could also explain why the experimental molecular weights of the heterodimer (89,000) and of the dimer (72,000) were in agreement with the theoretical molecular masses, assuming a 36-kDa Aes. Finally, Kanaya *et al.* (2) reported a molecular mass for recombinant Aes of 38 kDa by using a Sephacryl matrix.

The SELDI-TOF MS analysis revealed also the presence of a dimer of α-galactosidase in the 89-kDa peak. This should represent a minor component, because no increase of the 50-kDa band at the start of the 89-kDa peak was observed (Fig. 2).

In the Complex Aes Is Activated and α-Galactosidase Is Inactive—Although the α-galactosidase within this 1:1 complex with Aes is present in its inactive monomeric form (see "Results"), Aes activity is increased 6-fold. The effect is only on V_{max}, whereas K_m for substrate is unaffected. We also observed Aes activation due to protein dimerization, yet Aes homodimer, and activation associated with it appeared to be destroyed by low concentration (500 mM) of urea. In contrast, activation associated with Aes/α-galactosidase complex proved to be resistant to urea (see "Results" and Fig. 2*B* and Table I). These

results could indicate specificity in the Aes/α-galactosidase interaction and non-specificity in the formation of Aes dimers (see below).

It has been reported that the *E. coli* α-galactosidase is active in its tetrameric form and it requires magnesium ions and NAD for activity (32). We observed that NADH can substitute NAD in this role (no activity was observed in the absence of NADH). In this behavior the *E. coli* α-galactosidase is similar to other enzymes of the family 4 of glycosylhydrolases. NAD(H) is not transformed by the enzyme, and its cellular concentration could represent another point of control of α-galactosidase activity. Lastly, transcription of the *mel* operon, which comprises *mel*A and *mel*B genes coding for the α-galactosidase and a melibiose permease, respectively, is controlled by the product of *mel*R gene, a transcription activator requiring melibiose (raffinose) in the medium (31). Thus the metabolism of melibiose is under strict positive control through MelR and perhaps other pathways (such as the interaction with Aes demonstrated here), in a way similar to the control of maltose and maltodextrin metabolism through MalT.

A Unique Mechanism for MalT and α-Galactosidase Inhibition—Both the finding described here and the model we propose indicate that the Aes protein can repress the pathways for maltose and raffinose degradation through its protein-protein interaction with MalT and the α-galactosidase, respectively. It is worth noting that, in both cases, the mechanism is the same, *i.e.* Aes binding prevents oligomerization and therefore activation of MalT or α-galactosidase. In both cases, the effect represses carbohydrates metabolism. In contrast, the Aes protein is activated due to the interaction with α-galactosidase, and it is also likely that it could be induced in particular conditions that are still to be identified. Unfortunately, we have no indication about the Aes activity when interacting with MalT. Peist *et al.* (1) postulated the existence of a repressor for Aes that could be evocative of a transcriptional control in response to particular conditions. Because overexpression of Aes allows *E. coli* growth on minimal medium supplemented with triacetylglycerol as unique carbon source (1), a *scenario* is plausible in which the metabolic pathways for using sugars are repressed, if the bacterium uses triacetylglycerol or a similar ester as carbon source for growth. As a matter of fact, when incubated on minimal medium containing raffinose as sole carbon source, *E. coli* growth is reduced, following induction (IPTG) of Aes expression (Fig. 6). Moreover, high esterase activity is associated with low or undetectable α-galactosidase activity. These data support the *in vivo* role of the interactions between Aes and α-galactosidase we first identified *in vitro*. Furthermore, we controlled the specificity of this interaction by adding a small amount of purified Aes to crude extracts containing α-galactosidase activity. The α-galactosidase activity decreased indicating that the Aes/α-galactosidase interaction is able to counteract the monomer-monomer interactions in the pre-existing tetramer. Finally, the esterase activity increased when pure Aes was added to crude extracts containing the active

α-galactosidase, although not at the same level observed in the isolated complex (data not shown).

In conclusion, we report here the first evidence of a physical interaction of Aes protein with α-galactosidase in *E. coli*. This interaction appears to have strong physiological consequences, allowing us to propose a potential central role for Aes at the crossing of pathways for carbohydrate and lipid metabolism. To the best of our knowledge, this is also the first protein factor that is able to negatively control two different metabolic pathways at two different levels, namely at the expression (of *mal* genes) or activity (of the *mel*A gene product α-galactosidase) level.

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The Aes Protein and the Monomeric α-Galactosidase from*Escherichia coli* Form a Non-covalent Complex: IMPLICATIONS FOR THE REGULATION OF CARBOHYDRATE METABOLISM

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