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Subunit Homologies in Bacterial Luciferases*

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ABSTRACT: The tryptic peptides of the nonidentical subunits (α and β) of luciferase from both *Photobacterium fischeri* (Pf) and a second strain (MAV) were compared to evaluate possible homologies.

Three identical small peptides were found in one of the subunits from each of the strains.

This relationship, together with previous supporting evidence, led to a relabeling of the subunits of MAV, so that homologous pairs from the two strains are given the same Greek letter designation.

Bacterial luciferase from *Photobacterium fischeri* (Pf) is a bioluminescent protein which contains two nonidentical peptide chains (α and β) each with a molecular weight of about 4×10^4 (Friedland and Hastings, 1967). Recently, luciferase has been isolated from a different strain of luminous bacteria (designated as strain MAV) and likewise shown to contain two nonidentical polypeptide chains of similar molecular weights (Hastings *et al.*, 1969). Furthermore, neither of the luciferase chains from MAV was identical with those from Pf. This was deduced from amino acid compositions, electrophoretic mobilities, molecular weights, and complementation experiments. However, since both luciferases utilize the same substrates, it might be expected that certain sequences from the different polypeptide chains would be identical, especially for those residues essential for enzyme activity. In order to investigate possible homologies, tryptic maps of the individual subunits were compared. These experiments led to the identification of two dipeptides, Gly-Arg and Met-Lys, and more importantly, a tetrapeptide, Gly-(Trp,Gln)-Arg in one of the subunits from each strain.

The subunits from both strains were originally assigned α and β designations on the basis of their relative electrophoretic mobilities in 8 M urea (Hastings *et al.*, 1969). At that time there were indications (molecular weight and extinction coefficient) that Pf α-MAV β and Pf β-MAV α might be the more closely homologous pairs. Strong evidence in support of this relationship has been obtained in this work and we have therefore relabeled the MAV subunits so that the homologous pairs are given the same Greek letter designation. Thus, MAV α (formerly MAV β) is homologous with Pf α and MAV β (formerly MAV α) is homologous with Pf β.

Materials and Methods

Materials. Bacterial luciferase from Pf and MAV was prepared as described previously (Hastings *et al.*, 1965, 1969). The separated polypeptide chains (α and β) of both Pf and MAV were prepared by chromatography on DEAE-cellulose in 8 M urea as described by Friedland and Hastings (1967). The respective fractions were pooled and the urea removed by dialysis. All chemicals were reagent grade unless otherwise noted.

Tryptic Digestion. The separated polypeptide chains were dialyzed into 0.05 M *N*-ethylmorpholine (pH 8.0) to give a partially soluble suspension. The absorbance at 280 nm of the protein solutions was determined by dissolving an aliquot of the suspension in 8 M urea. Prior to digestion with trypsin, the protein (approximately 10 mg/ml) was incubated for 30 min in a boiling-water bath. This was found to be necessary to achieve complete tryptic digestion of the native enzymes. The solutions were then cooled to room temperature and L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone trypsin was added at a ratio of 1 mg/50 *A* units at 280 nm (as determined in 8 M urea). The solution was incubated at 37° for 5 hr, a small amount of insoluble matter removed by centrifugation, and the digestion terminated by freezing the solution.

Peptide Maps. The tryptic digests were electrophoresed on Whatman No. 1 paper (0.5 mg/cm) in pH 6.5 buffer (100 ml of pyridine–3 ml of glacial acetic acid–879 ml of water) for 45 min at 3000 V. The resulting sample was then stitched onto a second sheet of Whatman No. 1 paper; electrophoresis in pH 1.8 buffer (2% formic acid–8% acetic acid) was con-

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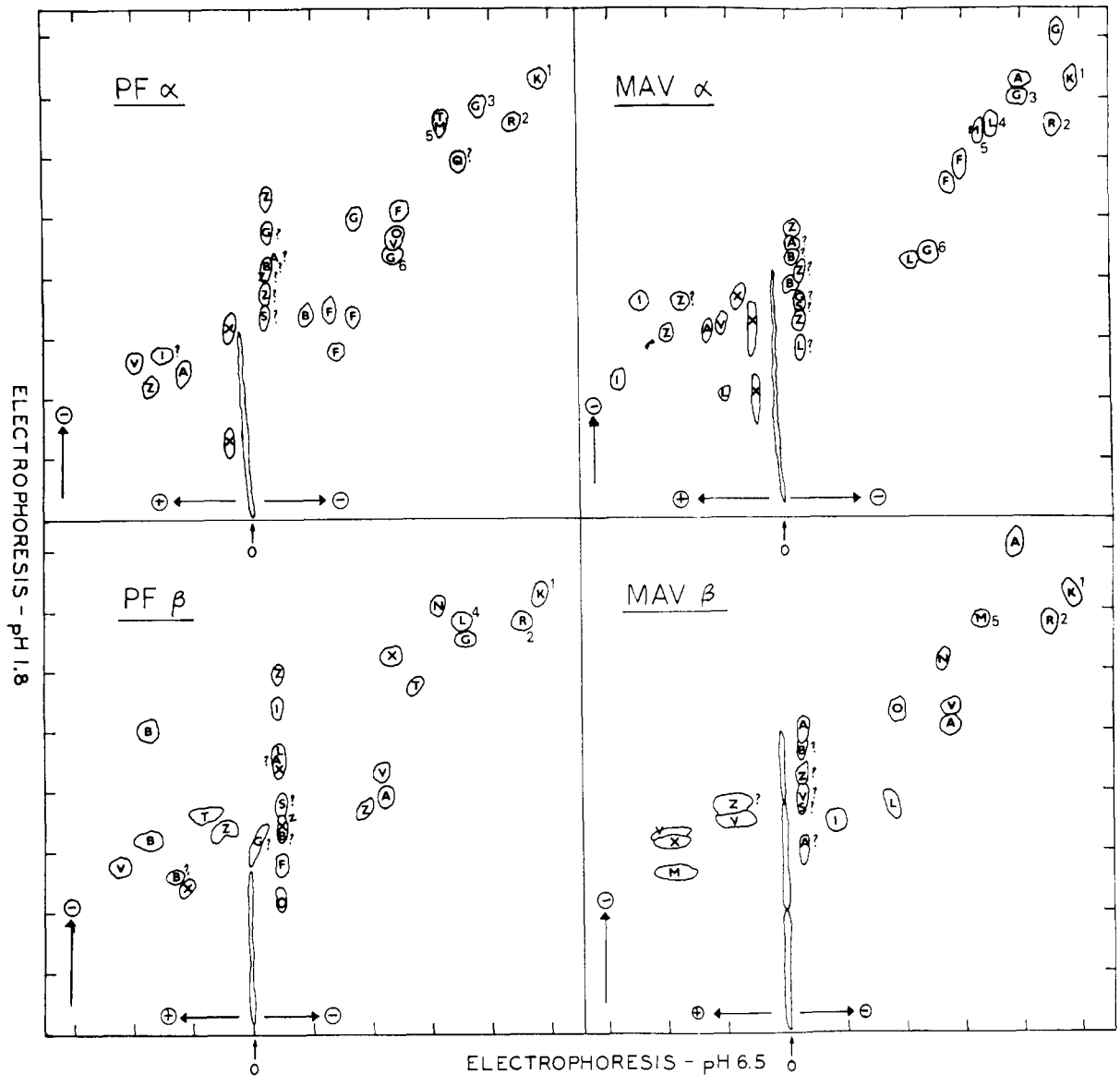

FIGURE 1: Peptide maps of the subunits, Pf α, Pf β, MAV α, and MAV β. The tryptic digests were electrophoresed at pH 6.5 in one direction, at pH 1.8 in a second, and then stained with ninhydrin as described in Methods. The amino-terminal residue of each tryptic peptide is indicated by a single capital letter (Eck and Dayhoff, 1966). A question mark indicates those peptides in which there was some uncertainty concerning the identity of the amino residue. Identical peptides present in more than one polypeptide chain are labeled 1–6. Peptide 1 was shown to be lysine and thus provides an internal standard for each map.

ducted for 45 min at 3000 V perpendicular to the initial electrophoresis. In some cases, the neutral peptides were cut out, stitched to a third sheet of Whatman No. 1 paper, and electrophoresis conducted in pH 3.5 buffer (5 ml of pyridine–50 ml of glacial acetic acid–895 ml of water) parallel to the initial electrophoresis. The peptide maps were then stained with a cadmium–ninhydrin reagent prepared by mixing 100 ml of 1% ninhydrin and 15 ml of cadmium acetate solution (5 g of cadmium acetate–250 ml of glacial acetic acid–500 ml of water).

Amino Acid Compositions. Amino acid analyses were conducted on a Beckman Model 120C AutoAnalyzer. Samples were hydrolyzed in 6 N constant-boiling HCl under vacuum for 20 hr at 110°.

Amino-Terminal Determinations. Samples (about 0.005 μmole) were reacted with dansyl chloride in a 1:1 mixture of acetone and water containing 0.05 M $NaHCO_3$ for about 2 hr at 37° (Gray, 1967). The samples were dried, hydrolyzed in 6 N HCl for 20 hr at 110°, and again taken to dryness. The dansylated amino acids were identified by thin-layer chromatography on polyamide sheets (Woods and Wang, 1967).

Results

Previous experiments by Hastings *et al.* (1969) showed that MAV luciferase contained two nonidentical subunits assigned α and β designations on the basis of their relative electrophoretic mobilities in 8 M urea. The subunits of MAV luci-

ferase have now been relabeled to conform to the indicated homology with the subunits of Pf luciferase; in the experiments presented below the new assignments will be used.

Figure 1 gives the peptide maps of Pf α, Pf β, MAV α and MAV β, as well as the N-terminal amino acid of each tryptic peptide. Approximately 25–30 ninhydrin-positive spots can be detected for each polypeptide chain out of a total of 30–35 expected tryptic peptides. The number of neutral peptides was estimated with the aid of an additional electrophoresis at pH 3.5, subsequent to the two electrophoresis steps given in Figure 1. The amino-terminal residue of each tryptic peptide was elucidated after isolation of the respective peptides by preparative electrophoresis.

A comparison of the tryptic maps clearly shows that only a few of the peptides (1–6) from any pair of luciferase chains have identical mobilities and identical N-terminal residues. As may be noted, these peptides are small and positively charged. None of the negatively charged peptides appear to be identical by these criteria. The results with the neutral peptides are not as clear but the results presented in Figure 1 combined with subsequent electrophoresis experiments at pH 3.5 indicate that at the most, only one or two of these peptides might be identical in any pair of peptide chains.

Amino acid compositions were performed on peptides 1–6 for each polypeptide chain in which they occurred. In each case, tryptic peptides with the same mobilities isolated from different chains were found to have identical amino acid compositions. These results enabled the sequences of these six short peptides to be established. Table I gives the sequence of peptides 1–6 and indicates which polypeptide chains contain the respective tryptic peptides.

Although lysine and arginine are present in the tryptic digest of each luciferase chain, this result does not lead to any conclusions regarding homologies between the different subunits. However, the presence of the dipeptides, Gly-Arg and Met-Lys, and in particular, the tetrapeptide, Gly-(Trp,Gln)-Arg in the tryptic digest of both Pf α and MAV α (formerly MAV β) strongly suggests that Pf α and MAV α are homologous. No extensive homology, as judged by the presence of identical tryptic peptides, can be observed between any other pair of luciferase polypeptide chains. The evidence for homology between the subunits of MAV and Pf obtained from analysis of the tryptic maps of the separated subunits, together with earlier results on the molecular weight and absorption properties (Hastings *et al.*, 1969) of the subunits led to the revised nomenclature for MAV subunits used in the present experiments.

TABLE I: Identical Tryptic Peptides in Different Luciferase Chains.[a]

Peptide	Sequence	Luciferase Chain: Pf α	Pf β	MAV α	MAV β
1	Lys	+	+	+	+
2	Arg	+	+	+	+
3	Gly-Arg	+	−	+	−
4	Leu-Lys	−	+	+	−
5	Met-Lys	+	−	+	+
6	Gly-(Trp,Gln)-Arg	+	−	+	−

[a] The presence or absence of the tryptic peptides is indicated by a plus or minus, respectively. Tryptophan was detected by staining the peptide maps with *p*-dimethylaminobenzaldehyde. In addition, its degradation products were detected in the amino acid analysis.

Discussion

The comparison of tryptic maps is generally useful for polypeptide chains which are closely related since small differences in amino acid sequence can lead to widely different peptide maps. For example, approximately 40% of the residues of the α and β chains of hemoglobin are identical (Braunitzer *et al.*, 1964) and yet the only common tryptic peptide is lysine (Guidotti *et al.*, 1962). The polypeptide chains of Pf and MAV luciferase have substantial differences in amino acid compositions (Hastings *et al.*, 1969). Thus, it might be expected that even if different luciferase subunits were homologous, none of the tryptic peptides would be identical.

The discovery of the two dipeptides and (of even greater significance) a tetrapeptide, common to the α subunits of both Pf and MAV constitutes strong evidence that these subunits are homologous. Such a result also infers that the β subunits of the two bacterial strains are closely related. These conclusions are supported by experimental evidence on the molecular weights and ultraviolet absorption properties of the α and β subunits of the two bacterial strains (Hastings *et al.*, 1969). At present, it is not possible to decide whether this result indicates a very extensive degree of homology between the α subunits of MAV and Pf or whether these peptides are particularly resistant to mutation, perhaps because they may be essential for enzyme activity. Further experiments with mutant luciferases that are inactive may be valuable in this regard.

Evidence for extensive homology between the α and β subunits was not detected in the present experiments although lysine and arginine were present in the tryptic digest of each luciferase subunit. However, such a result certainly does not eliminate the possibility that the α- and β-polypeptide chains are themselves related and perhaps originated by gene duplication. If this supposition is correct, the present experiments suggest that such a gene duplication arose prior to the divergence of the two bacterial strains. Such a supposition also would suggest that each of the nonidentical polypeptide chains has the same function. Hybridization experiments and active-site studies are currently in progress to determine the functional properties of the α and β subunits. It will be interesting to elucidate if bacterial luciferase can be classified as a heteropolymeric protein with homologous subunits such as hemoglobin (Braunitzer *et al.*, 1964), or as a multifunctional enzyme complex such as tryptophan synthetase (Crawford and Yanofsky, 1958).

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Isolation and Characterization of Rabbit Muscle Triose Phosphate Isomerase*

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ABSTRACT: Rabbit muscle triose phosphate isomerase has been purified to homogeneity as judged by analytical ultracentrifugation and disc gel electrophoresis. The enzyme has a molecular weight of 52,900 by sedimentation equilibrium, 56,000 by amino acid composition, and 53,000 by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. Data from carboxypeptidase digestion of the enzyme are consistent with two identical subunits having a C-terminal sequence of -(Val,Asp,Phe,Ile)-Asn-Ile-Ala-Lys-Gln. Electrofocusing of triose phosphate isomerase reveals the presence of two major and two minor species having similar specific activities.

Recent studies (Hartman, 1970a,b) on the active site of rabbit muscle triose phosphate isomerase (D-glyceraldehyde 3-phosphate:ketol isomerase, EC 5.3.1.1) required large amounts of the enzyme. The difficulty in obtaining sufficient quantities by a previously published method (Czok and Bücher, 1960) and the heterogeneity of commercial material prompted the development of the isolation procedure described in our present report. The purified enzyme has been characterized with respect to amino acid composition, molecular weight, and subunit structure.

Materials and Methods

Frozen, deboned muscle tissue from the back and hind legs of young (8–12 weeks) rabbits was purchased from Pel-Freez Biologicals, Inc. NADH, DL-glyceraldehyde 3-phosphate, α-glycerophosphate dehydrogenase, and rabbit muscle triose phosphate isomerase were obtained from the Sigma Chemical Co. Rabbit muscle triose phosphate isomerase was also obtained from Boehringer Mannheim Corp. Carboxypeptidase A was a product of Worthington Biochemical Corp. Ammonium sulfate (Ultra Pure biological grade) was purchased from Schwarz BioResearch, Inc.

Protein Concentration. The method of Lowry *et al.* (1951) with human serum albumin as the standard or spectrophotometric measurements at 280 nm were used to determine protein concentrations. The $\epsilon_{1\,cm}^{1\%}$ at 280 nm of purified triose phosphate isomerase was determined as described below.

Enzyme Assays. Triose phosphate isomerase was assayed at 24° by the method of Beisenherz (1955). The assay mixture (3 ml) contained 0.15 mM NADH, 1 mM DL-glyceraldehyde 3-phosphate, 28 μg of α-glycerophosphate dehydrogenase, 0.3 mM EDTA, and 20 mM triethanolamine hydrochloride (pH 7.9). The reaction was initiated by the addition of a quantity of triose phosphate isomerase such that the change in A per minute at 340 nm did not exceed 0.2. One unit of activity is defined as the conversion of 1 μmole of glyceraldehyde 3-phosphate into dihydroxyacetone phosphate per min and represents a decrease in A_{340nm} of 2.07 optical density units per min.

Extinction Coefficient. Two samples of triose phosphate isomerase at about 8 mg/ml were dialyzed exhaustively (three changes during 48 hr) against 0.01 M ammonium acetate (pH 6.4). Aliquots of the dialyzed samples were diluted 20-fold into various buffers (0.05 M Tris-hydrochloride, pH 7.5; 0.1 M sodium phosphate, pH 6.0, 7.0, and 8.0; 0.1 M sodium cacodylate, pH 6.0). The A at 280 nm of these solutions were identical. Samples (3 ml) of the undiluted, dialyzed protein solutions were lyophilized to dryness in preweighed vials, redissolved in 5 ml of water, and again lyophilized. The vials were then heated in a vacuum desiccator to constant weight, from which the $\epsilon_{1\,cm}^{1\%}$ of the two samples was calculated as 13.07 and 13.18, respectively. Values of 11.0 and 12.1 have been reported for commercial rabbit muscle triose phosphate isomerase (Hartman, 1968; Coulson *et al.*, 1970).

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