PHYSICOCHEMICAL ANALYSIS OF A PROTEIN WITH NERVE GROWTH ACTIVITY FROM THE MUSCLE TISSUE OF Rana ridibunda

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A protein nerve growth factor has been isolated in the pure form from the muscles of the lake frog Rana ridibunda. The individuality of the substance obtained has been shown, its molecular mass (106.0-110.0 kDa) and pI value (9.2-9.4) have been determined, and lysine and valine have been found as the N- and C-terminal amino acid residues of its polypeptide chain. In view of the detection of the presence of sugars (about 1%), the substance has been identified as a glycoprotein. Its absorption spectra in the UV region has been obtained and the amino acid composition of the isolated protein has been determined. Information on the amino acid composition of various proteins with nerve growth activity has been subjected to comparative analysis with the aid of computer programs, and on this base their phylogenetic "tree" has been constructed.

Nerve growth factors (NGFs) – protein regulators of the growth and differentiation of sympathetic and embryonic sensitive neurons – have been detected in various organs of fish, reptiles, and mammals [1-3]; unfortunately, there is no information in the literature on the NGFs of amphibia. In a study of the properties and action of NGFs there are difficulties connected with their identification as nerve growth factors, since known methods based on an analysis of functional activity (biological effect) and immunological properties (cross-reactivity) do not always give reliable information [1, 3]. At the present time, a large amount of information has accumulated on neurotrophic factors possessing NGF activity but not belonging to this group of proteins [4-6]. On the other hand, the idea of a multiplicity of different NGFs [7] is regarded critically by many authors. Convincing proofs that a protein belongs to the NGF group can probably be given only by its detailed physicochemical characterization and also a careful analysis of its reception and the mechanism of its action [8]. It is clear that the answer to these questions requires the preliminary isolation of an NGF in the pure form.

We have succeeded in purifying a protein from the muscles of the lake frog Rana ridibunda which possesses nerve growth activity according to the results of a biotest on a culture of the spinal ganglia of chick embryos. In the present paper we give results characterizing its physicochemical properties.

The material with nerve-growth activity obtained from the muscle tissue of the lake frog was characterized by protein heterogeneity. The results of its electrophoretic separation are shown in Fig. 1. A typical axon-stimulating effect in a concentration of 20-25 ng/ml was caused by electrophoretic fractions 1 and 2, and when fraction 1 was subjected to repeat electrophoresis, in addition to material remaining at the start, active material corresponding to fraction 2 was found. This creates the impression that fraction 1 always contained two components, one of which with respect to its relative electrophoretic mobility was identical with a component of fraction 2. The results of electrophoresis, isoelectric focussing, and the analysis of N- and C-terminal amino acid residues showed the protein homogeneity of the material of fraction 2, and we identified it as the protein with nerve growth activity. It may be assumed that the molecule of the protein sought has a complex organization and can dissociate into active subunits, so that in solution a dynamic equilibrium is always set up between the dissociated and the initial forms. The initial complex may be fairly stable; its breakdown is therefore only slight but increases in the presence of detergents and reducing agents, as we in fact see in the SDS electrophoresis of fraction 2 (Fig. 2).

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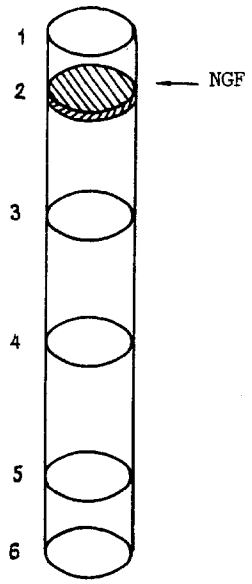

Fig. 1

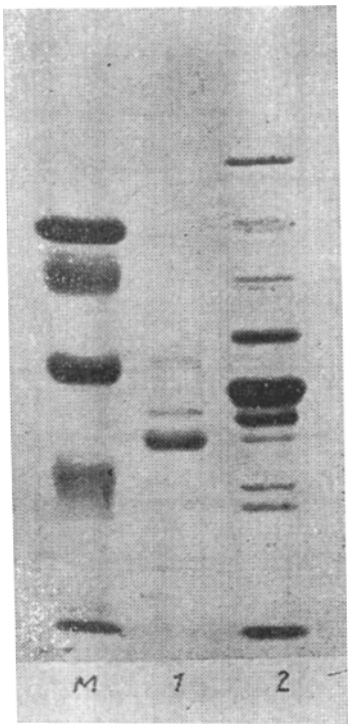

Fig. 2

Fig. 1. Preparative electrophoresis of the protein fraction of the NGF (PS) in polyacrylamide gel (7.5% PAAG), Tris-glycine buffer, pH 8.3, electrophoresis for 180 min at a current strength of 30 mA/tube): 1-6) electrophoretic fractions; the arrow shows the NGF zone.

Fig. 2. Comparative SDS electrophoresis of the purified NGF from lake frog muscle (1) and an extract of the muscle tissue (2) in the presence of markers (M) (12% PAAG, Tris-glycine buffer, pH 8.6, containing 1% of SDS; electrophoresis for 240 min at a current strength of 30 mA): 1) immunoglobulin, MM 67 kDa; 2) bovine serum albumin, MM 67 kDa; 3) ovalbumin, MM 43 kDa; 4) lactalbumin MM 18 kDa.

During the experimental proof of the purity of the protein isolated (fraction 2), information characterizing its physicochemical properties was obtained. Thus, in the isoelectric focussing of fraction 2 in Ampholines in the pH interval of 3.5-9.5 it was possible to establish the isoelectric point (pI) of the protein, which was 9.2-9.4. This agrees well with literature reports [9] showing the basic properties of NGFs of different origins, including a high-molecular-mass NGF from the placenta. An estimate of the molecular dimension of the muscle protein with the aid of ultracentrifugation gave a KS value of 7.4 and a molecular mass of 106-110 kDa. The same results were obtained when the protein that we were investigating was subjected to column gel filtration (Fig. 3). This corresponds to the molecular dimensions of the 7S NGF complex which, as is known, includes 2α-2γ-β subunits [1]. However, other of our results did not confirm the structure of the complex in the protein isolated. Thus, the detection of a glycine and a valine residue at the N- and C-ends, respectively, was evidence in favor of the assumption that we had isolated not a complex but an individual protein of large dimensions. A high-molecular-mass NGF has also been detected in fibroblasts L 929, muscle-cell cultures, and other materials [9-11].

The molecular dimensions of these proteins are 314.0 ± 9.0 kDa for α-cells, 106.0 kDa for primary muscle cells and the lines of myogenic cells, and 150.0 kDa for the cotyledons of the placenta. At the same time, in the corresponding publications arguments are given in favor of the assumption that these high-molecular-mass NGFs are protein complexes that are stable in dilute solutions [11]. It may be assumed that we also obtained a fairly stable

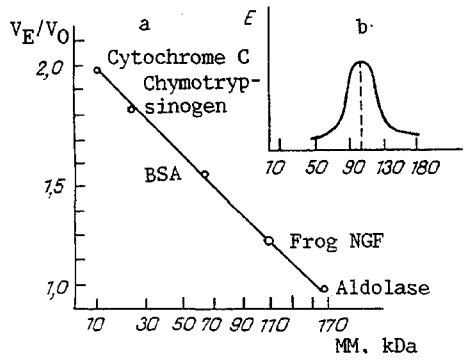

Fig. 3. Determination of the molecular mass of the NGF from lake frog muscle: a) graph of the gel filtration of the NGF on Sephadex C-100; b) ratio of the molecular dimensions of the substance and its elution volumes.

TABLE 1. Amino Acid Composition of a Protein with Nerve Growth Activity from Lake Frog Muscles (in moles of amino acid per mole of protein)

Amino acid	Time of hydrolysis, h			Number of residues
	24	48	72	
Aspartic acid	70,01	70,1	70,3	70
Threonine	37,7	37,9	37,6	38
Serine	45,0	45,3	45,1	45
Glutamic acid	72,2	72,35	72,1	72
Proline	20,5	20,3	20,1	20
Glycine	128,9	128,6	128,8	129
Alanine	56,6	56,8	56,7	57
1/2-Cystine	49,8	49,7	46,9	50
Valine	49,9	49,6	49,65	50
Methionine	17,4	17,1	17,0	17
Isoleucine	30,9	30,8	30,6	31
Leucine	39,9	39,5	39,8	40
Tyrosine	19,7	19,8	19,6	20
Phenylalanine	20,6	20,5	20,9	21
Lysine	29,1	29,8	29 05	30
Histidine	101,8	101,8	101,85	102
Arginine	17,8	17,9	17,7	18
Tryptophan	18,8	18,7	18,9	19
Total				828
Molecular mass: from the results of ultracentifugation and column gel filtration				106.0
from amino acid composition				103,4

protein complex in which the individual subunits had identical or blocked ends. The absence from the protein that we were studying of proteolytic and BAEE-esterase activities, which are exhibited in known NGF complexes by the α- and, possibly, the β-subunits [1] may confirm this hypothesis.

The protein (or protein complex) isolated from lake frog muscles had a characteristic absorption spectrum in the UV region with a maximum at 284 nm and a minimum at 250 nm. From the coefficient of molecular extinction we calculated the amount of tryptophan (19 residues), and the whole amino acid composition of the substance isolated is given in Table 1.

The molecular mass calculated from the composition given in the Table agrees satisfactorily with the results of our other investigations; the slight discrepancy can be explained by the fact that the material investigated contained about 1% of sugars. On amperometric titration no free SH groups were found in the protein, i.e., the molecular structure was fairly rigidly stabilized by 25 disulfide bonds.

The protein that we had isolated was compared with respect to its amino acid composition with others, including proteins accurately identified as NGFs, and also other factors possessing other functional properties together with nerve growth activity. Special programs that we had developed previously [12] were used, but certain refinements were introduced into the amino acid composition of the protein under investigation and the list of substances compared was broadened. On the basis of the results obtained, we have constructed a phylogenetic "tree" (Fig. 4) modeling the process of evolution of such proteins and demon-

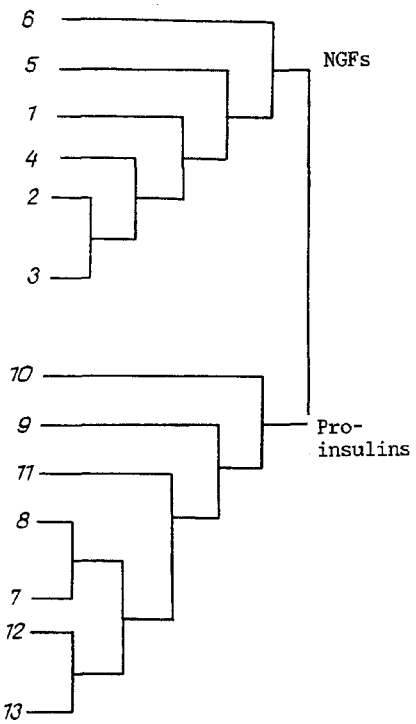

Fig. 4. Phylogenetic tree of proteins of the NGF and proinsulin family: NGFs: 1) mouse; 2) man; 3) cattle; 4) chick; 5) snake; 6) frog. Proinsulins: 7, 8) rat; 9) horse; 10) duck; 11) man; 12) cattle; 13) pig.

strating the degrees of their relationship. The proteins compared divide clearly into two groups – nerve growth factors and proinsulins from various animals.

In the evolution of these protein structures from the pra-form (precursor) a divergence accompanied by functional specialization and the appearance of the corresponding structural features probably took place. The protein with nerve growth activity from the muscular tissue of the lake frog falls into the NGF group where it occupies an isolated position like the NGFs of snakes, mice, and chicks. The two mammalian NGFs (bovine and human) exhibit considerable structural similarity; in this connection the separate position of the murine NGF, which in a preceding analysis [12] was closer to mammalian MGFs, remains unclear.

EXPERIMENTAL

In this work we used particles of quartz glass with dimensions of 0.3-0.6 mm^3 and Sephadexes from Pharmacia (Sweden), UM-10 ultrafilters from Amicon (USA), kits for electrophoresis from Reanal (Hungary) and for isoelectric focussing from LKB (Sweden), a standard test mixture of marker proteins from Serva (Germany), polyamide plates from Schleicher and Schüll (Germany), and carboxypeptidase Y from Sigma (USA). The other reagents were of domestic production and of KhCh. ["chemically pure"] or ChDA ["pure for analysis"] grade. The frog Rana ridibunda was collected in the spring and autumn and was maintained in baths in water at a temperature of +5-10°C. All the chromatographic experiments and also electrophoresis and isoelectric focussing were carried out with the aid of known procedures [13, 14].

The molecular mass of the protein was determined by column gel filtration and by analytical ultracentrifugation. Ultracentrifugation was conducted in IKhRV AN RUz [Institute of the Chemistry of Plant Substances of the Uzbekistan Republic of Sciences] on an MOM-3170 instrument (Budapest, Hungary). The N-terminal acid residue was identified in the form of the DNS derivative [15] with the aid of chromatography on polyamide plates [16], and the C-terminal residue also by chromatography after the hydrolysis of the protein with carboxypeptidase. After hydrolysis with twice-redistilled hydrochloric acid, the amino acid composition of the protein was investigated on a Biotronic IC 7000 amino acid analyzer (FRG).

Absorption spectra in the UV region were taken on a UV-VIS spectrometer. The tryptophan content was calculated on the basis of molar extinction coefficients [17]. The presence of sulfhydryl groups was judged from the results of amperometric titration [18]. The computer programs for comparing proteins with respect to their amino acid compositions had been developed and tested previously [12], and the phylogenetic "tree" was constructed with the aid of the VOSTORG program kindly provided by Yu. A. Sprizhitskii (IMG AN SSSR [Institute of Molecular Genetics of the USSR Academy of Sciences]). The amount of protein in the material was determined by Lowry's method [19] and sugars by Dubois' method [20].

Proteolytic activity was evaluated from the hydrolysis of casein [21], and BAEE esterase activity by the method of Schwert and Takenaka [22]; nerve growth activity was investigated on the spinal ganglia of 7- to 9-day chick embryos, which were cultivated by the "rotating test-tube" method [23].

The ganglia, placed on cover-slips with a collagen coating, were immersed in test-tubes each containing 25 ml of culture medium consisting of Eagle's medium, medium 199, Hanks' solution, and inactivated cattle serum taken in a ratio of 32:32:32:4 (V/V) and were incubated with dilutions of the material under investigation at 37°C for 18-24 h.

The axon-stimulating effect was evaluated on Fenton's five-point scale [24] followed by recalculation of the activity to biological units (BUs).

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