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On the Relationship of Zinc Ion to the Structure and Function of the 7S Nerve Growth Factor Protein†

Scott E. Pattison and Michael F. Dunn*

ABSTRACT: The 7S nerve growth factor (7S NGF) is an oligomeric protein consisting of three distinct classes of subunits, α, β, and γ (A. P. Smith, S. Varon, and E. M. Shooter (1968), *Biochemistry 7,* 3259). The β subunit contains the growth promoting activity while γ is a potent esteropeptidase. The proteolytic activity of γ is virtually completely inhibited in the 7S NGF aggregate (L. A. Greene, E. M. Shooter, and S. Varon (1969), *Biochemistry 8,* 3735). In this paper, we report that divalent metal ion chelating agents effect a seven- to tenfold increase in the esteropeptidase activity of 7S NGF at pH 7.40. Plots of esteropeptidase activity vs. chelator concentration give saturation curves which are either sigmoidal (EDTA) or hyperbolic (*o*-phenanthroline) depending on the chemical structure of the chelator. A survey of common divalent metal ions shows that only zinc ion ($K_i = 8 \times 10^{-7}$ *M*) and, to a lesser extent, cadmium ion are effective, reversible inhibitors of both 7S NGF and the γ subunit esteropeptidase activities. We have found that during isolation of 7S NGF, Zn^{2+} is selectively associated with the oligomer in a ratio of approximately 1–2 g-atoms of zinc/mol of 7S NGF with an apparent affinity which is orders of magnitude tighter than is indicated by the K_i value for the γ subunit. Dialysis to pH 4.0 where 7S NGF is known to undergo a reversible dissociation (A. P. Smith, S. Varon, and E. M. Shooter (1968), *Biochemistry 7,* 3259) brings about a tenfold reduction in the zinc ion content of the protein. This reduction is reversed on dialysis back to pH 7.4. In contrast, the isolated subunits contain only trace amounts of zinc ion at pH 7.4. Preliminary metal ion exchange experiments indicate that, of the common metal ions known to substitute for zinc in other zinc-metalloproteins, only cadmium ion is effective in substituting for zinc ion in 7S NGF. The fact that zinc ion is specifically bound to native 7S NGF, and that the zinc ion content of the system is critically dependent on the subunit aggregation state strongly suggests that zinc ion is an integral structural component of native 7S NGF.

The in vivo growth and the differentiation of two neural crest derivatives, the noradrenergic neurons of the superior cervical ganglia and the sensory neurons of the dorsal root ganglia, are dependent upon, and are regulated by, the nerve growth factor (NGF)[1] protein (Schenkein, 1972). Two forms of NGF have been isolated from the adult male mouse submaxillary gland: a 25,000 mol wt species (2.5S NGF) composed of two identical polypeptide chains (Angeletti and Bradshaw, 1971), and a 140,000 mol wt/species (7S NGF) composed of three electrophoretically distinct classes of subunits, α, β, and γ. The 2.5S NGF is either identical with, or a close derivative of, the β subunit (Perez-Polo et al., 1972a). Both 7S and 2.5S NGF possess similar in vitro growth stimulating activities (Schenkein, 1972). Frazier et al. (1972) have suggested a common ancestral gene for 2.5S NGF and proinsulin on the basis of sequence similarities between the two. The three γ isozymes present in 7S NGF exhibit potent esteropeptidase activities with high specificity for the hydrolysis of α-*N*-acyl-L-arginine esters and amides (Greene et al., 1969). This activity is greatly diminished in the 7S NGF-subunit complex. No well-defined biological activity has been found for the α class of subunits.

There are many unanswered questions concerning the in vitro roles of 7S NGF vs. 2.5S NGF, or the β subunit. The fact that the esteropeptidase activity of the γ enzyme is virtually completely inactive in the 7S NGF oligomer implies that the 7S species, as isolated, is a precursor of the physiologically functional species. Since the 2.5S species is approximately as active as 7S NGF in promoting neurite outgrowth in the in vitro organ culture assay, the physiological role of 7S NGF is unclear. It has been shown, however, that the binding domains between 7S NGF subunits are highly specific, and that 7S NGF appears to be the molecular form

† From the Department of Biochemistry, University of California, Riverside, California 92502. *Received August 9, 1974.* Support of this work by American Cancer Society Grant No. DT-4 (National division) and by Grant No. 544 (California division), and by funds from the University of California Cancer Research Coordinating Committee is gratefully acknowledged.

[1] Abbreviations used are: NGF, nerve growth factor; BAPNA, α-*N*-benzoyl-DL-arginine-*p*-nitroanilide.

of NGF present in the submaxillary gland (Perez-Polo et al., 1972b).

Although 7S NGF has been suggested to be a pentamer with the composition of $\alpha_2\beta\gamma_2$, the subunit combining stoichiometry has not been established (Bamburg et al., 1971). The interrelationships between subunit structure and subunit function for 7S NGF remain largely unknown terrain. Moore et al. (1974) have proposed that suppression of the γ-esteropeptidase activity within the 7S NGF species arises from interactions between the active site of γ and the C-terminal arginyl residue of the β subunit.

The fact that the biological properties of an oligomeric protein, almost without exception, are drastically altered by a change in subunit aggregation state (Monod et al., 1965; Matthews and Bernhard, 1973; Malhotra et al., 1974) almost without question means that the specific subunit interactions which make up the oligomer also contribute to the biological function of the oligomer. It is often found that small molecules play a key role in maintaining the structural and/or functional integrity of the oligomer. Divalent metal ions have been found to be important in either or both of these capacities for many protein complexes (Nelbach et al., 1972; Scott, 1934; Griffin et al., 1973; Blundell et al., 1972; Schlesinger, 1965).

This work is part of a long range effort to investigate the molecular details of the process of neuroblast growth and differentiation through the identification and investigation of the functional roles played by the 7S NGF protein and its subunits in this process. In this paper we show: (a) the specific presence of zinc ion in 7S NGF, (b) that incubation of 7S NGF with divalent metal ion chelators at high ionic strength promotes both the dissociation of 7S NGF to lower molecular weight species and the concomitant activation of γ-esteropeptidase activity, and (c) that zinc and cadmium ions are potent inhibitors of the esteropeptidase activities of γ and of 7S NGF.

Materials and Methods

The 7S NGF preparations used in this study were isolated from the submaxillaries of Swiss-Webster adult male mice (>35 g) by the method of Varon et al. (1967). The individual subunits were isolated from 7S NGF via the procedure of Smith et al. (1968). The molecular properties of these proteins and the growth activities were identical with those reported by these authors. Concentrated stock solutions of 7S NGF were stored at 0°, dilute solutions of 7S NGF and solutions of the subunits were stored at 5°. All protein manipulations were carried out at 5°. Protein concentrations have been estimated from the following extinction coefficients (taken from Smith, 1969): 7S NGF, 0.9 OD_{280} ml/mg; α, 1.1 OD_{280} ml/mg; β, 0.7 OD_{280} ml/mg; γ, 1.1 OD_{280} ml/mg.

The esteropeptidase activities of 7S NGF and the γ subunit were determined from the rate of hydrolysis of the chromophoric substrate, α-*N*-benzoyl-D-L-arginine-*p*-nitroanilide (BAPNA) (Sigma Chemical Co.). All assays were conducted at 25 ± 0.2° in 0.05 *M* Tris buffer (Sigma) (pH 7.40). The rate of *p*-nitroaniline production was monitored at 410 nm with a Beckman DB-GT spectrophotometer. In the standard assay used in these experiments, a 10-μl aliquot of a 0.1 *M* stock solution of BAPNA (in dimethyl sulfoxide) was added to a cuvet containing 1.00 ml of buffer. The kinetic run was then initiated by adding a microliter aliquot of the protein to be assayed to the cuvet and recording the time course of the optical density change at 410 nm. Additional reagents (e.g., chelators, metal ions) were added in microliter amounts either prior to, or subsequent to, the addition of protein (enzyme) as called for in a particular experiment. (The specific details of each assay are listed in the appropriate figure captions, see Results.) The γ-subunit preparation was found to have a specific activity of 0.6 μm/(min mg) in this assay.

Dialysis experiments generally were carried out at 5.0° over a time period of 24 hr in 0.05 *M* Tris buffer (pH 7.40). The dialysis tubing (Union Carbide Corp.) was prepared for use by first boiling with $NaHCO_3$ and EDTA for 15 min followed by extensive washing with glass double-distilled water according to the method of Zeppezauer (1971).

Sedimentation velocity ultracentrifugation runs were made with a Beckman Model E analytical ultracentrifuge equipped with an AN-D rotor. The runs were carried out at 5° with a rotor speed of 59,780 rpm and with protein concentrations of approximately 5 mg/ml.

The zinc content was directly measured using a Perkin-Elmer Model 303 atomic absorption spectrophotometer. Total metal ion content was determined using a Tarrell-Ash 3.4 m direct reading emission spectrograph.

Results

Effects of Divalent Metal Ion Chelators on 7S NGF. The discovery which led to the present work is described by the experiments depicted in Figure 1. As shown in Figure 1A, the addition of ethylenediaminetetraacetic acid (EDTA) to a solution of 7S NGF causes a large (ca. tenfold) increase in esteropeptidase activity. This increase occurs on a time scale which is slow relative to the steady-state rate of BAPNA turnover. Indeed, the activation time-course is similar to that previously reported for an activation process thought to be dependent on dissociation of the 7S complex to lower molecular weight species (Greene et al., 1969).

Note that the activity of γ (Figure 1B) is unaffected by the addition of EDTA. Other chelators (e.g., *o*-phenanthroline (OP), 8-hydroxyquinoline, 8-hydroxyquinoline-5-sulfonic acid, 2,2′-dipyridine, nitrilotriacetic acid, *N*-methyliminodiacetic acid, and 1,2-*trans*-cyclohexanediaminetetraacetic acid) produce similar activation of 7S NGF esteropeptidase activity (S. E. Pattison and M. F. Dunn, in preparation).

For a given chelator, both the extent of activation and the rate-of-change of the (steady-state) esteropeptidase activity are dependent on the concentration of the chelator (Figure 2) and the concentration of 7S NGF (S. E. Pattison and M. F. Dunn, in preparation). At high chelator concentrations, the extent of activation saturates. The 7–10-fold increase in esteropeptidase activity obtained under these conditions approximates that of the free γ subunit. Thus, these findings strongly imply: (a) that a divalent metal ion plays an important role in the subunit structure of 7S NGF, since divalent metal chelators effect a seven- to tenfold increase in esteropeptidase activity, and (b) that since activation has been reported to accompany subunit dissociation (Greene et al., 1969), activation caused by chelators similarly may involve dissociation of 7S NGF to lower molecular weight species.

Preliminary analytical ultracentrifuge velocity studies indicate that the addition of high EDTA concentrations (80–150 m*M*) effects the dissociation of the native 7S NGF oligomer to one (or more) species exhibiting an $s_{20,w}$ value of ~4 S (S. E. Pattison and M. F. Dunn, unpublished results).

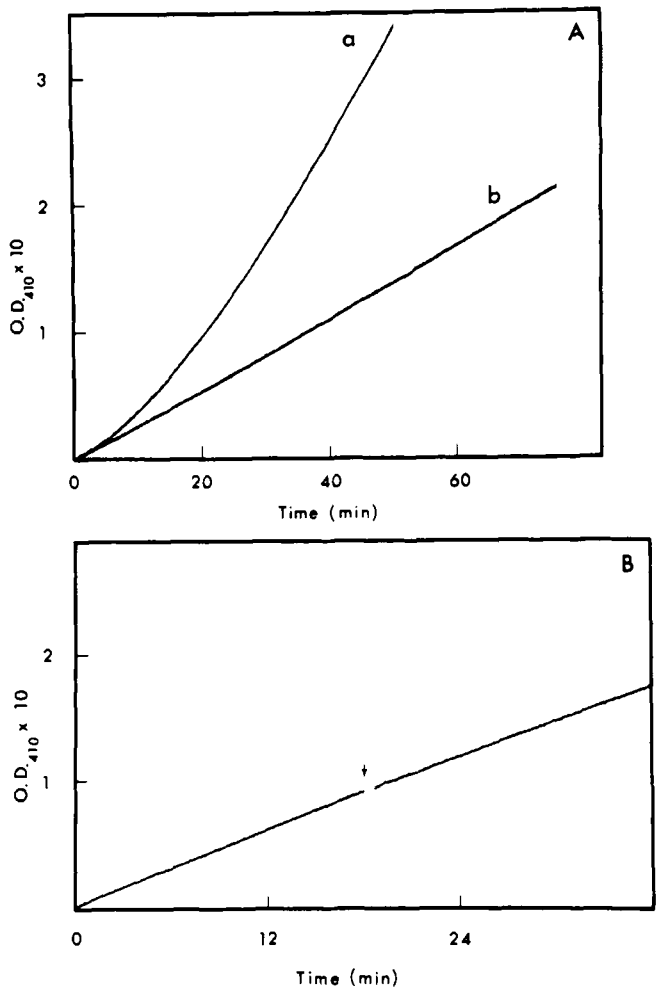

FIGURE 1: Comparison of the effects of EDTA on the hydrolysis of BAPNA by 7S NGF and by the γ subunit. Enzyme activities were assayed by adding a microliter amount of concentrated enzyme to a cuvet containing BAPNA, 1 mM, in 0.05 M Tris buffer (pH 7.40) at 25 ± 0.2°. (A) Trace (a) shows the initial time course for the hydrolysis of BAPNA by ~0.2 μM 7S NGF in the presence of 0.1 mM EDTA. Trace (b) shows the initial time course for the hydrolysis of BAPNA under the same conditions in the absence of EDTA. (B) This trace shows the initial time course for the rate of hydrolysis of BAPNA by ~0.2 μM γ subunit. As indicated by the vertical arrow, 10 μl of 10.0 mM EDTA was added to the reaction mixture to give a final EDTA concentration of 0.1 mM approximately 18 min after the initiation of the assay. Note that the rate of BAPNA hydrolysis is not affected by the presence of EDTA.

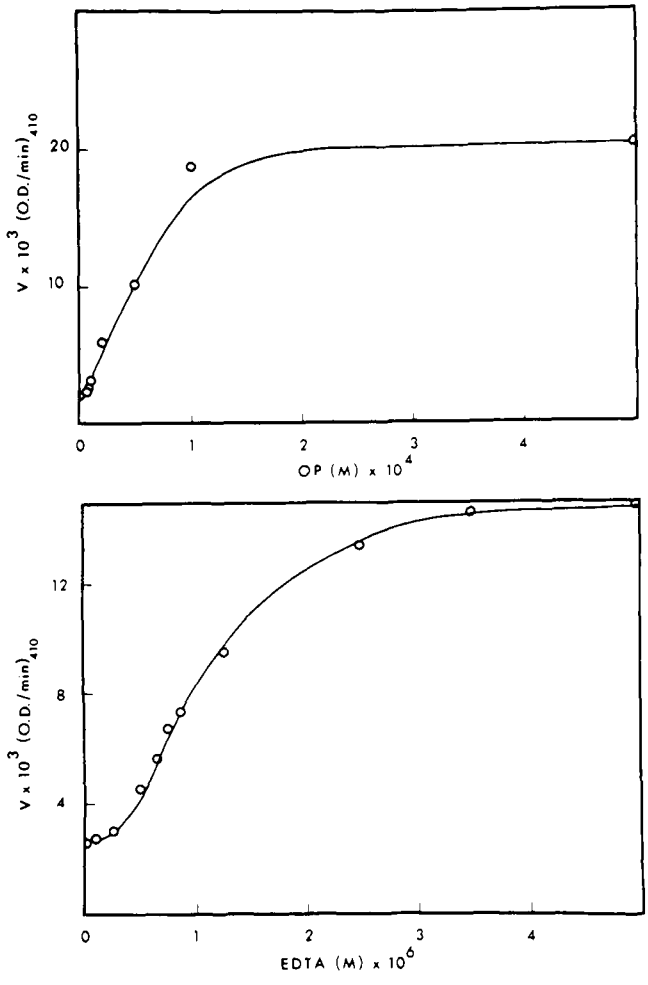

FIGURE 2: The dependence of 7S NGF esteropeptidase activity on chelator concentration. Samples containing 7S NGF, ~0.25 μM, and variable amounts of chelator in 0.05 M Tris buffer (pH 7.40) were incubated: (A) at 25° for 1 hr with variable concentrations of o-phenanthroline (OP), and (B) at 5° for 24 hr with variable concentrations of ethylenediaminetetraacetic acid (EDTA). At the conclusion of the incubation, 10 μl of 0.1 M BAPNA was added (final concentration, 1.0 mM) to the sample at 25 ± 0.2° and the rate of BAPNA hydrolysis (v) was measured by monitoring the rate of change of optical density at 410 nm.

The Effects of Zinc Ion on the Esteropeptidase Activities of 7S NGF and the γ Subunit. The γ subunit, when isolated from 7S NGF, exhibits linear, steady-state kinetics during hydrolysis of the artificial substrate, BAPNA. As indicated above (Figure 1), the presence of EDTA has no effect on the activity of γ-subunit preparations. A survey of the effects of common divalent transition and alkaline-earth metal ions on the activity of γ is summarized in Table I. This summary shows that only zinc ion and cadmium ion significantly inhibit γ-esteropeptidase activity under these conditions. The time-course shown in Figure 3A demonstrates that Zn^{2+} inhibition occurs on a time scale which is rapid in comparison to the mixing time. Plots of $1/V$ vs. zinc ion concentration (Figure 3B) indicate a competitive mode of inhibition ($K_i \simeq 8 \times 10^{-7}$ M).

When concentrated 7S NGF is diluted into the assay mixture, the rate of BAPNA hydrolysis shows a characteristic lag before the attainment of a pseudo-zero-order steady-state rate (Greene et al., 1969). Greene et al. postulate that this lag phase arises from partial dissociation of the 7S NGF complex. A survey of the common divalent transition and alkaline-earth metal ions (Table I) again indicates that zinc ion is the most effective inhibitor of esteropeptidase activity. 7S NGF stock solutions which have been preincubated with EDTA show a susceptibility to Zn^{2+} inhibition which is quantitatively similar to Zn^{2+} inhibition of γ (Figure 4).

Zn^{2+} Content of 7S NGF and Its Subunits. Since the above noted inhibition by Zn^{2+} and Cd^{2+} implies a highly specific interaction between γ and metal ion, it was of interest to investigate the divalent metal ion content of native 7S NGF. Atomic emission spectrograph analysis shows a significant presence of the following metals: Ca, Mg, Fe, Cu, Zn, Cr, Ba. The presence of stoichiometrically significant amounts of Zn^{2+} is confirmed by atomic absorption measurements (see Table II and Figure 5).

The atomic absorption data summarized in Table II and in Figure 5 unambiguously demonstrate that a significant fraction of the zinc ion present in the supernatant from the gland homogenate copurifies with 7S NGF during isolation.

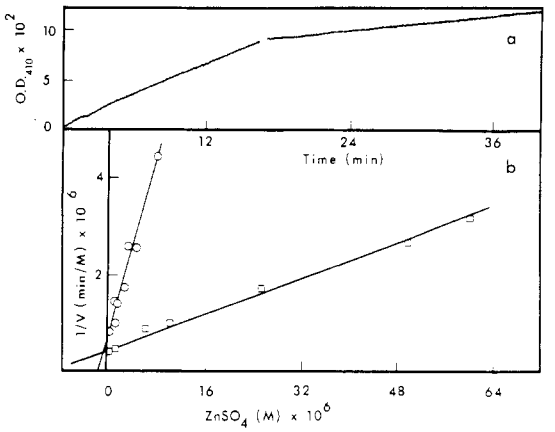

FIGURE 3: The inhibition of γ subunit esteropeptidase activity by zinc ion. The inset (a) shows the initial time course for the γ subunit catalyzed hydrolysis of BAPNA in the absence of zinc ion. Note that the addition of 10 μl of 1 mM $ZnSO_4$ (final concentration, 10 μM) at the vertical arrow brings about the "instantaneous" inhibition of BAPNA hydrolysis. (b) The effect of zinc ion concentration on the rate of BAPNA hydrolysis (v) is plotted as $1/v$ vs. the concentration of zinc ion according to the treatment of Dixon (1953). Conditions: γ subunit, ~0.2 μM; BAPNA, (□) 1 mM, (○) 0.1 mM; 0.05 M Tris buffer (pH 7.40) and 25 ± 0.2°.

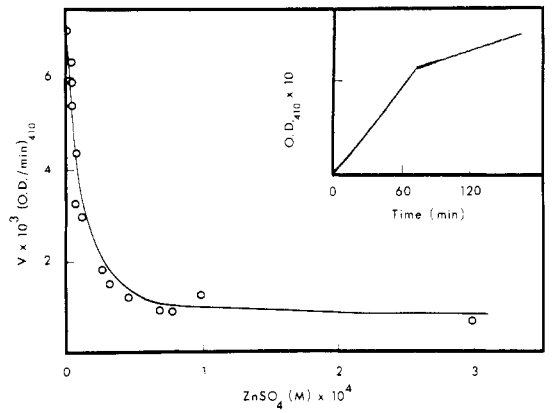

FIGURE 4: The inhibition of 7S NGF esteropeptidase activity by zinc ion. The effect of zinc ion concentration on the rate of BAPNA hydrolysis (v) is plotted as v vs. the concentration of zinc ion. Conditions: 7S NGF, ~0.2 μM; BAPNA, 1 mM; 0.05 M Tris buffer (pH 7.40), and 25 ± 0.2°. Since the 7S NGF oligomer undergoes partial dissociation when diluted, 7S NGF samples were preincubated at 25° for 1 hr prior to the initiation of the assay by the addition of BAPNA. The inset shows the time course for the 7S NGF catalyzed hydrolysis of BAPNA initially in the absence of zinc ion. Note that the addition of 10 μl of 1 mM $ZnSO_4$ (final concentration 10 μM) at the vertical arrow brings about the "instantaneous" inhibition of BAPNA hydrolysis.

The 7S NGF isolation procedure (Varon et al., 1967) involves two gel filtration steps (G-100 and G-150 Sephadex) and a step involving separation by charge (DE-11). The 7S NGF subunits are isolated by ion exchange chromatography over a CM-32 column. As is evident in Figure 5, in every purification step the fractions containing 7S NGF also contain the highest zinc ion concentrations. Note in Figure 5 that the final step in the 7S NGF isolation procedure, G-150 Sephadex gel filtration, produces a nearly homogeneous peak of 7S NGF that chromatographs coincident with the zinc ion peak. The zinc ion content of 7S NGF ranged from approximately 1.3 to 1.9 g-atoms of Zn^{2+}/mol of 7S NGF from six separate isolations.

Upon dialysis of 7S NGF to pH 4.0 (Table II) the zinc ion content is greatly reduced. Under these conditions the

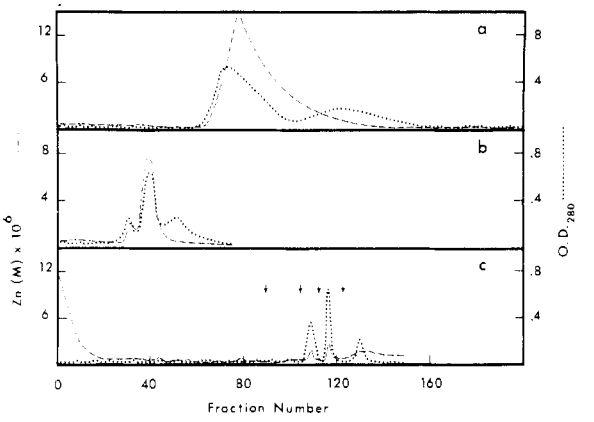

FIGURE 5: Comparison of zinc elution profiles with protein $OD_{280\,nm}$ profiles for the column chromatographic steps involved in the 7S NGF isolation procedure of Varon et al. (1967) and the subunit isolation of Smith et al. (1968). The profiles in (a), (b), and (c) compare the zinc elution pattern (- - -) as determined by atomic absorption spectroscopy with the $OD_{280\,nm}$ elution profile (·· ·) as a function of the eluent fraction. (a) G-100 Sephadex chromatography of the gland homogenate. The fractions enriched with 7S NGF run slightly behind the first protein peak and are coincident with the first zinc peak eluted from the column. (b) G-150 Sephadex chromatography of the 7S NGF fraction from the DEAE-cellulose chromatography step (see Table II). (The major peak eluted from this column contains the highly purified 7S NGF used throughout this work.) (c) CM-32 cellulose chromatography of highly purified 7S NGF from the G-150 Sephadex chromatography step. From left to right, the protein peaks eluted from this column contain purified α, γ, and β subunits. The column is initially washed with 0.05 M sodium acetate buffer (pH 4.0) containing 0.15 M sodium chloride and 1 mM EDTA. The vertical arrows indicate (beginning from left to right): first arrow, continuation of column washing but without EDTA; second arrow, application of 7S NGF; third arrow, shift of eluent buffer sodium chloride concentration to 0.4 M; fourth arrow, change of eluent buffer to 0.05 M glycine (pH 9.40) plus 2.0 M NaCl. The fraction sizes were (a) 5.3 ml, (b) 5.3 ml, (c) 1.4 ml.

Table 1: Divalent Metal Ion effects on the Esteropeptidase Activity of 7S NGF and the γ Subunit.

Divalent Metal Ion Salts	Initial Rate ($OD_{410\,nm}$/min) γ Subunit[a]	7S NGF[b]
Control (no added metal ion)	0.033	0.0074
$CuSO_4$	0.030	0.0067
$MgCl_2$	0.035	0.0070
$CaCl_2$	0.035	0.0072
$MnCl_2$	0.033	
$CoCl_2$	0.037	0.0067
$ZnSO_4$	0.012	0.0006
$CdSO_4$	0.021	

[a] The effects of divalent metal ions on the γ subunit esteropeptidase activity were assayed via the following procedure: 25 μl of a solution of 1 mM divalent metal ion in 0.05 M Tris buffer (pH 7.4) was added to 1 ml of the same buffer containing ~0.4 μM γ subunit; 10 μl of 0.1 M BAPNA was added to initiate the assays and esteropeptidase activity was monitored by measuring the rate of increase in OD at 410 nm at 25 ± 0.2°. [b] The effects of divalent metal ions on 7S NGF esteropeptidase activity were assayed via a procedure similar to the one used with the γ subunit; 25 μl of a solution of 1 mM divalent metal ion in 0.05 M Tris buffer (pH 7.4) was added to 1 ml of the same buffer containing ~0.1 μM 7S NGF; 10 μl of 0.1 M BAPNA was added to initiate the assay, and the initial rate of reaction was monitored at 410 nm.

7S NGF complex dissociates to lower molecular weight species (Varon et al., 1968). When the mixture is dialyzed back to pH 7.4 with buffer prepared from glass double-distilled water, all of the zinc is regained (Table III) and the

Table II: Zinc Content in Protein Fractions Isolated during the Purification and Isolation of 7S NGF and Its Subunits.[a]

Isolation Step and Conditions	$Zn^{2+} \times 10^6 M$	OD_{280}	$Zn^{2+} \times 10^6 M / OD_{280}$	g-atom of Zn^{2+}/mol of 7S NGF
I. Fractions from DE-11 column				
0.05 *M* Tris (pH 7.4)	0.52	0.18	2.90	
0.05 *M* Tris (pH 7.4), 0.01 *M* NaCl	0.68	0.13	5.25	
0.05 *M* Tris (pH 7.4), 0.08 *M* NaCl	2.90	0.20	14.50	2.03
0.05 *M* Tris (pH 7.4), 2.00 *M* NaCl	3.56	0.70	5.20	
2.00 *M* NaCl				
II. Pressure concentration step (7S NGF from DE-11 column)				
Concentrate (0.05 *M* Tris (pH 7.4)–0.08 *M* NaCl)	61.5	4.90	12.60	1.76
Eluent	2.8	0.12	23.40	
III. Pooled 7S NGF fraction from G-150 column	3.6	0.30	12.00	1.68
IV. Pressure concentration step (G-150 Sephadex fraction containing 7S NGF)				
Concentrate	30.6	2.60	11.80	1.65
Eluent	1.4	0.03	46.50	
V. Pressure concentrated G-150 fraction dialyzed to pH 4.0 (0.05 *M* sodium acetate)	2.8	2.20	1.30	0.18
				g-atom of Zn^{2+}/mol of Subunit
VI. Subunit Fractions (CM32 column)				
α (0.05 *M* acetate (pH 4.0)–0.15 *M* NaCl)	0.48	0.20	2.34	0.067
α 0.05 *M* Tris (pH 7.4))	0.78	0.18	4.35	0.12
γ (0.05 *M* acetate (pH 4.0)–0.40 *M* NaCl)	0.64	0.27	2.36	0.051
γ (0.05 *M* Tris (pH 7.4))	1.25	0.25	5.00	0.11
β (0.05 *M* glycine (pH 9.4)–2.0 *M* NaCl)	1.32	0.08	16.50	0.29
β (0.05 *M* Tris (pH 7.4))	0.48	0.095	5.00	0.09

[a] 7S NGF was isolated via the procedure of Varon et al. (1967). The subunits of 7S NGF were isolated via the procedure of Smith et al. (1968). Briefly, the isolation of 7S NGF from male mouse submaxillary glands involves column chromatographic steps utilizing: (1) size separation over G-100 Sephadex, (2) charge separation over DEAE-cellulose, and (3) size separation over G-150 Sephadex. The subunits of 7S NGF were isolated via column chromatography over carboxymethylcellulose at pH 4.0. Under these pH conditions, 7S NGF dissociates to subunits. The zinc content of the fractions was measured by atomic absorption spectrometry. The accuracy of the zinc determinations is ±0.5 μM zinc ion. The ratios, g-atom of Zn^{2+}/mol of 7S NGF and g-atom of Zn^{2+}/mol of subunit, are calculated from the experimentally determined OD_{280} values using the extinction coefficients derived from Smith (1969), see Materials and Methods, assuming the samples contain pure 7S NGF and pure subunits, respectively. Thus, the values are only approximate.

Table III: Effect of Competition between Zinc Ion and Other Metal Ions on the Level of Zinc Ion in 7S NGF.

Sample and Dialysis Conditions	$Zn^{2+} \times 10^6 M$	OD_{280}	$Zn^{2+} \times 10^6 M / OD_{280}$	g-atom of Zn^{2+}/mol of 7S NGF
A. Dialysis in 0.05 *M* Tris buffer (pH 7.4)[a]				
Stock 7S NGF	3.20	0.34	9.42	1.31
7S NGF dialyzed against 0.05 *M* Tris (pH 7.4)	3.05	0.33	9.25	1.29
7S NGF dialyzed against 0.05 *M* Tris (pH 7.4)–0.1 m*M* $CdSO_4$	1.32	0.33	4.0	0.56
7S NGF dialyzed against 0.05 *M* Tris (pH 7.4)–0.1 m*M* $CuSO_4$	2.65	0.33	8.04	1.12
7S NGF dialyzed against 0.05 *M* Tris (pH 7.4)–0.1 m*M* $CoCl_2$	2.48	0.32	7.75	1.08
B. Dialysis in 0.05 *M* acetate buffer, pH 4.0, followed by dialysis in 0.05 *M* Tris buffer (pH 7.4)[b]				
Stock 7S NGF	3.20	0.34	9.42	1.31
7S NGF dialyzed against 0.05 *M* acetate (pH 4.0)	0.85	0.32	2.70	0.38
7S NGF (pH 4.0) dialyzed against 0.05 *M* Tris (pH 7.4)–0.1 m*M* $CuSO_4$	2.10	0.35	6.00	0.84
7S NGF (pH 4.0) dialyzed against 0.05 *M* Tris (pH 7.4)–0.1 m*M* $CoCl_2$	2.40	0.35	6.85	0.96

[a] Ten milliliters of a solution of ~2 μM 7S NGF in 0.05 *M* Tris buffer (pH 7.4) was dialyzed overnight at 5° against 500 ml of the same buffer containing additional metal ions as indicated in the table. Dialysis tubing was pretreated as given in the Materials and Methods section. The accuracy of the zinc ion determinations is ±0.5 μM zinc ion. [b] Ten milliliters of a solution of ~2 μM 7S NGF in 0.05 *M* Tris buffer (pH 7.4) was dialyzed overnight at 5° against 0.05 *M* sodium acetate buffer (pH 4.0) in order to remove zinc ion. The 7S NGF was then dialyzed against 500 ml of 0.05 *M* Tris buffer (pH 7.4) containing the added metal ions as indicated in the table.

protein exhibits physical and chemical properties identical with native 7S NGF. Since dialysis to pH 7.4 restores the zinc lost at pH 4.0, 7S NGF must scavenge trace amounts of zinc from the dialysate.

Subunits isolated by CM-32 chromatography of the pH 4.0 solution (Table II, Figure 5) contain only trace amounts of zinc ion. Dialysis of the individual subunits to pH 7.4 does not significantly change their zinc content. The zinc ion content of the buffers used in these experiments is below the detection limits of the atomic absorption spectrophotometer ($\leq 5 \times 10^{-7} M$).

Divalent Metal Ion Exchange with Zinc in 7S NGF. Preliminary dialysis experiments were undertaken to test the specificity of the Zn^{2+}–7S NGF interaction. In these experiments 7S NGF was dialyzed either from pH 7.4 to pH 4.0 and back to pH 7.4, or at pH 7.4, in the presence of a 500-

fold excess of the divalent metal ion. Of the metals tested (Table III), only dialysis in the presence of cadmium brings about a significant decrease in the zinc content of 7S NGF. Note that dialysis against cadmium ion for an additional 24-hr period brings about no further decrease in the zinc ion content of 7S NGF.

Discussion and Conclusions

These studies unambiguously demonstrate that native 7S NGF contains 1–2 g-atoms of zinc/mol of protein. During the final isolation step, both 7S NGF and zinc ion move through the G-150 Sephadex column as single, coincident peaks (Figure 5). In proceeding isolation steps, those column fractions which contain 7S NGF also contain high zinc ion concentrations. Since the isolation scheme consists of several steps involving separation both by charge and by size, it is highly improbable that the zinc ion associated with 7S NGF is an artifact which arises from the isolation procedure.

The avidity and specificity with which native 7S NGF binds zinc ion are indicated by the observation that dialysis to pH 4.0 effects the dissociation of 7S NGF and the removal of zinc ion from the protein, and that this zinc is fully regained when the sample is dialyzed back to pH 7.4 against buffers containing only trace amounts of zinc ion ($\leq 5 \times 10^{-7}$ *M*). The high degree of specificity for zinc ion is emphasized by the finding that 7S NGF is highly efficient in scavenging trace amounts of zinc ion from the buffer in these dialysis experiments even when high concentrations of other divalent metal ions (e.g., Cu^{2+}, Co^{2+}, Cd^{2+}) are present during dialysis back to pH 7.4 (see Table III). Thus, of the divalent metal ions tested, only dialysis in the presence of 10^{-4} *M* cadmium ion noticeably diminishes the zinc ion content of 7S NGF. Since the attainment of equilibrium in each case has not been rigorously established, specificity could be a manifestation of kinetic control rather than thermodynamic control. In any case, these experiments indicate that the interaction between 7S NGF and zinc ion is highly specific regardless of the origins of specificity.

In contrast to the behavior of 7S NGF, the isolated subunits do not contain stoichiometrically significant amounts of zinc ion at neutral pH. Quantitation of the zinc ion content of the individual subunits during isolation is complicated by the divalent metal binding capacity of the CM-32 carboxymethylcellulose column. Zinc ion is eluted from this column under the same conditions of ionic strength which elute the γ subunit. Nevertheless, this zinc is removed by gel filtration or by dialysis at pH 7.4.

It has been established by Greene et al. (1969) that the γ-esteropeptidase activity is virtually completely suppressed in the 7S NGF oligomer. Dissociation of the 7S aggregate under mild conditions—e.g., high dilution, exposure to high ionic strength, or exposure to pH's above 8 or below 5—brings about a concomitant activation of γ. Our work demonstrates that a similar (albeit more extensive) activation of 7S-esteropeptidase activity takes place when 7S NGF is incubated with divalent metal ion chelators. This finding suggests that the esteropeptidase activity associated with 7S NGF preparations is critically influenced by the presence of a divalent metal ion. Furthermore, the fact that the zinc ion content of the system is critically dependent on the subunit aggregation state suggests that zinc ion is a necessary structural component of native 7S NGF.

The unusually high specificity manifest in the zinc ion inhibition of γ-esteropeptidase activity is intriguing. The specificity of this interaction, see Table I, suggests that zinc ion plays a role in the suppression of γ-esteropeptidase activity within the 7S oligomer. In any case, it is clear that the active 7S oligomer corresponds to a more highly constrained ("taut") quaternary structure (Monod et al., 1965), since elimination of specific interprotomer bonding forces via dissociation greatly enhances γ activity.

For other protein systems, subunit aggregation has been shown to depend on the presence of zinc. Both the aggregation of aspartate transcarbamylase catalytic and regulatory subunits in the native enzyme (Nelbach et al., 1972) and the aggregation of insulin protomers (a possible genetic relative of NGF) to the hexamer (Scott, 1934) depend critically on specific interprotomer liganding to zinc ion. In the case of aspartate transcarbamylase, the zinc-protein binding domain, in addition, is selectively important to the homotropic interactions between catalytic subunits (Griffin et al., 1973). In the case of insulin, the hexamer exists in dissociative equilibrium with free protomers under physiological conditions (Blundell et al., 1972). Zinc strongly shifts this equilibrium toward the hexameric state. This interaction is believed to affect storage and release of insulin in the β cells of the pancreas (Huber and Gershoff, 1973). Perhaps not surprisingly, zinc has proved to be important to the function of these protein systems while fulfilling a quaternary structural role. It can be concluded from the evidence presented in this paper that both the quaternary structure and the function of 7S NGF also are intimately related to the presence of zinc in the oligomer.

Acknowledgments

The authors are grateful to Drs. Silvio Varon and Ning Pon both for contributions in the form of theoretical discussions and for technical guidance during the initial stages of this project. We thank Mr. Charles Raiborn for his assistance in learning the standard biological assay for nerve growth factor activity. We are grateful to Dr. Anthony Norman for his assistance in using the atomic absorption spectrophotometer, and we thank Mr. Frank Moses for assisting with the in vitro biological activity assay.

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ATP ↔ P_i Exchange and Membrane Phosphorylation in Sarcoplasmic Reticulum Vesicles: Activation by Silver in the Absence of a Ca^{2+} Concentration Gradient†

Leopoldo de Meis* and Martha M. Sorenson‡

ABSTRACT: The activation of ATP ↔ P_i exchange, normally associated with a Ca^{2+} concentration gradient in sarcoplasmic reticulum vesicles, can be obtained in "leaky" vesicles in 4–10 m*M* $CaCl_2$. In the micromolar range, Ag^+ activates the ATP ↔ P_i exchange two- to fourfold. Similar concentrations of Ag^+ promote a parallel inhibition of Ca^{2+}-activated ATP hydrolysis and Ca^{2+} uptake in intact vesicles. Maximal inhibition of these activities by Ag^+ leaves the Mg^{2+}-dependent ATPase unaffected. No net synthesis of ATP was demonstrated in leaky vesicles. The effects of Ag^+ depend on the protein concentration and persist after removal of Ag^+ from the medium. Membrane phosphorylation from P_i or from ATP is respectively activated or inhibited by Ag^+ in reciprocal fashion.

Sarcoplasmic reticulum vesicles (SRV)[1] isolated from skeletal muscle actively take up Ca^{2+} from the medium in the presence of ATP and Mg^{2+}. Hydrolysis of ATP and the translocation of Ca^{2+} into the vesicles involve a transfer of the γ-phosphate of ATP to a membrane protein (E), forming an acylphosphoprotein (E~P). Accordingly, the following reaction sequence has been proposed (Makinose, 1969; Hasselbach, 1972).

$$E + ATP \xleftrightarrow{Ca^{2+}} E\sim P + ADP$$
$$E\sim P \longleftrightarrow E + P_i$$

Recently, it has been demonstrated that the entire process of Ca^{2+} transport can be reversed and that the enzymatic system of the SRV membrane is able to use the energy derived from a Ca^{2+} concentration gradient for the chemical synthesis of ATP (Barlogie et al., 1971; Makinose, 1971, 1972, 1973; Makinose and Hasselbach, 1971; Hasselbach et al., 1972; Panet and Selinger, 1972; Deamer and Baskin, 1972; Masuda and de Meis, 1973, 1974). The following data support this conclusion.

(a) *Net Synthesis of ATP.* When SRV previously loaded with calcium phosphate are incubated in a medium containing EGTA,[1] Mg^{2+}, ADP, and P_i, a fast release of Ca^{2+} coupled with ATP synthesis from ADP and P_i is observed (Makinose, 1972, 1973; Makinose and Hasselbach, 1971).

(b) *ATP ↔ P_i Exchange.* When intact SRV are incubated in a medium containing ATP, Mg^{2+}, [^{32}P]P_i, and Ca^{2+}, calcium phosphate is accumulated by the vesicles and a Ca^{2+} concentration gradient is built up until a steady state is reached in which a slow Ca^{2+} efflux is balanced by an ATP-driven influx. When this condition is reached, a steady rate of exchange between P_i and the γ-phosphate of ATP is observed (Makinose, 1971; Racker, 1972; de Meis and Carvalho, 1974). This exchange implies that the two reactions shown above are operating simultaneously forward (ATP hydrolysis) and backward (ATP synthesis from ADP and P_i).

(c) If the SRV are made "leaky" by various means, the Ca^{2+} concentration gradient is abolished and both the net synthesis of ATP and ATP ↔ P_i exchange reaction are arrested (Makinose, 1971; de Meis and Carvalho, 1974).

When SRV are loaded with calcium phosphate, the formation of a gradient coincides with the establishment of three conditions: a high Ca^{2+} concentration (in the millimo-

† From the Instituto de Biofísica, Universidade Federal do Rio de Janeiro, Centro de Ciências Médicas, Cidade Universitária, Ilha do Fundão, Rio de Janeiro, GB, Brazil. *Received January 8, 1975.* This investigation was supported in part by the Conselho Nacional de Pesquisas, Brasil (T.C. 17.117), by the Conselho de Ensino para Graduados da U.F.R.J., and by the Banco Nacional de Desenvolvimento Econômico (FUNTEC 241).

‡ Recipient of a fellowship from the Conselho Nacional de Pesquisas, Brazil. Present address: Laboratory of Neurophysiology, Department of Neurology, College of Physicians and Surgeons of Columbia University, New York, N.Y. 10032.

[1] Abbreviations used are: SRV, sarcoplasmic reticulum vesicles; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid.