# THE DIMER-MONOMER EQUILIBRIUM CONSTANT FOR [$^{125}$I]β NERVE GROWTH FACTOR

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SUMMARY. The equilibrium constant for [$^{125}$I]β nerve growth factor was determined using polyacrylamide gel electrophoresis to separate the monomer and dimer. Various concentrations of the radiolabelled nerve growth factor were incubated for 24 and 48 hours. The equilibrium constants obtained for both incubation periods were the same, $3.2 \pm 1.4 \times 10^{-11}$M and $2.6 \pm 1.6 \times 10^{-11}$M, respectively. Thus, at physiological concentrations the β nerve growth factor is in the dimeric form almost exculsively.

## INTRODUCTION

Nerve growth factor (NGF[1]) is necessary for the growth and development of the sympathetic and sensory nervous systems (1-3). The major source for this protein is the male mouse submaxillary glands (4). From this source, the NGF is isolated as a high molecular weight species, 7S NGF (5) or a low molecular weight species, βNGF (6). The βNGF is composed of two identical chains, held together by noncovalent forces, each with a molecular weight of 13,259 (7, 8). A question which needed to be answered was what is the biologically active species, whether it is the βNGF dimer, the monomer, or if both are biologically active? It was recently shown (9) that the dimer is biologically active and an attempt was made to show that the monomer was biologically active (10). Frazier, et al. (10) covalently linked 2.5S NGF to Sepharose beads in the presence of 6 M guanidine hydrochloride in an attempt to show that NGF acts through a receptor. With their method, they also

[1]NGF, nerve growth factor; 7S NGF, high molecular weight NGF, βNGF, the β-subunit of 7S NGF; 2.5S NGF, limited proteolytic degradation product of βNGF, fully biologically active; $\beta^1$NGF, βNGF with C-terminal arginines on both chains; $\beta^2$NGF, one C-terminal arginine; $\beta^3$NGF, no C-terminal arginines; ANS, 8-anilino-1-napthelene sulfonic acid, magnesium salt; TEMED, N,N,N',N'-Tetramethylethylene diamine; SDS, sodium dodecyl sulfate.

reportedly demonstrated that the monomer was biologically active. However, recent evidence (11-16) has indicated that care must be taken when performing studies with Sepharose-bound proteins. These studies put some doubt on the results obtained for receptor-protein interactions from proteins covalently bound to Sepharose beads.

An indirect method that may give an answer as to the biological activity of the monomer is to determine the monomer-dimer equilibrium constant for βNGF. Young, et al. (17) attempted to determine this equilibrium constant for 2.5S NGF. They determined an association constant for 2.5S NGF of approximately $10^7 M^{-1}$. This indicates that the monomer is also biologically active, since at physiological concentrations the NGF would be completely dissociated into monomer.

[$^{125}$I]βNGF has been used extensively for studying the action of NGF and its receptor (18-20). It was therefore of interest to determine the dissociation constant for [$^{125}$I]βNGF and see if it is the same as that determined for 2.5S NGF. The results given here show that [$^{125}$I]βNGF has an entirely different equilibrium constant than that reported for 2.5S NGF.

## MATERIALS AND METHODS

Isolation of βNGF. The βNGF was isolated by the method of Varon, et al. (6) from 7S NGF. The 7S NGF was isolated by the method of Varon, et al. (5) as modified by Wagner, et al. (21). After isolation, the βNGF was stored in 0.2% acetic acid at a concentration of 2 mg/ml.

Iodination of βNGF. βNGF was iodinated as described by Herrup and Shooter (18). βNGF (17.5 µl of a 2 mg/ml solution) was added to 1 mCi of [$^{125}$I]NaI (1.5 µl of a solution, pH 8-10), 6 µl of lactoperoxidase (1 mg/ml in phosphate buffer, pH 7.4, ionic strength 0.1) and 25 µl of 800 µM $H_2O_2$ in the same phosphate buffer were then added. The reaction mixture (50 µl total volume) was incubated at room temperature for one hour. The reaction was stopped by the addition of 50 µl of 0.4% acetic acid and incubated for an additional 10 minutes. Cytochrome c (100 µl of a 5 mg/ml solution in 0.4% acetic acid) was added to decrease the amount of "sticking" of βNGF.[2] This mixture was dialyzed extensively against 0.2% acetic acid until greater than 99% of the counts were precipitable by trichloroacetic acid (TCA precipitable counts) and stored at 4°C until used. There were approximately 1,100 counts per ng of βNGF. Samples were counted for either 10 minutes or until 100,000 counts were reached on a Nuclear-Chicago γ counter.

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[2]Without cytochrome c, 60-90% of the protein binds noncovalently (sticks) to glass, polyethylene, etc. whereas less than 1% "sticks" in the presence of cytochrome c (Rice and Stach, unpublished data).

Equilibration of Various [$^{125}$I]βNGF Solutions. [$^{125}$I]βNGF (approximately 3 to 100 ng/ml) was incubated at room temperature in a total volume of 300 µl in Beckman (Beckman Instruments, Fullerton, California) polyethylene microfuge tubes (with 2.5 mg/ml of cytochrome c to help prevent "sticking" of the βNGF) for 24 and 48 hours. The solutions were made approximately 12% in sucrose (100 µl of 50% sucrose solution) before electrophoresis.

Gel Electrophoresis. Various concentrations of [$^{125}$I]βNGF (approximately 3 to 100 ng/ml in 100 µl of solution) were analyzed on polyacrylamide gels using a continuous phosphate buffer system (pH 7.0 ionic strength 0.01). The gels (0.5 x 8 cm, 7.5% acrylamide, 0.2% bisacrylamide, 0.05% TEMED, 0.05% ammonium persulfate, in phosphate buffer, pH 7.0, ionic strength 0.04) were run at 7 ma per gel for two hours with the cathode at the bottom. Gels containing 100 µg βNGF and 100 µg lysozyme were stained by the method of Hartman and Udenfriend (22) with 8-anilino-1-napthelene sulfonic acid, magnesium salt (ANS). The fluorescence was observed with the use of a uv lamp and the gels containing the [$^{125}$I]βNGF were sliced in a position corresponding to the point between the lysozyme and βNGF (Fig. 1A, is a scan of the fluorescence observed from the lysozyme and βNGF, scanned on a Helena "Flur-Vis", Quick-Scan Densitometer, Beaumont, Texas).

Sodium doceyl sulfate (SDS) polyacrylamide gel electrophoresis was performed by the method of Stach and Shooter (9). [$^{125}$I]βNGF (164 ng) was analyzed on 12% SDS polyacrylamide gels, 0.5 x 8 cm (Fig. 1B) after incubation in 1% SDS for approximately 20 hours at room temperature. The gel was cut into 1 mm slices (44 total slices) and counted as above.

Concentration of [$^{125}$I]βNGF After Incubation. The concentration of [$^{125}$I]βNGF that remained in solution, after the appropriate incubation period, was determined using 100 µl aliquotes (separate from those used for electrophoresis) of the incubation medium before electrophoresis. The counts per minute obtained for the samples were divided by the counts per minute determined for the [$^{125}$I]βNGF stock solution (1,058 cpm/ng [$^{125}$I]βNGF), all counts are minus background (45 cpm).

Other Chemicals. Lysozyme and cytochrome c were purchased from Sigma Chemical Company, St. Louis, Missouri. ANS was purchased from Eastman, Rochester, New York, and recrystallized from hot water. [$^{125}$I]NaI, 1 mCi was purchased from New England Nuclear in a "V" vial in approximately 1 µl of solution, pH 8-10.

## RESULTS

The results given in Table I show that the equilibrium dissociation constant for the monomer-dimer equilibrium is $3.2 \pm 1.4 \times 10^{-11}$ M for incubation at 24 hours and an equilibrium constant of $2.6 \pm 1.6 \times 10^{-11}$ M for incubation at 48 hours. This gives an average equilibrium constant of $2.9 \pm 1.5 \times 10^{-11}$ M. These solutions were incubated in the presence of cytochrome c (2.5 mg/ml); this was to help to prevent the "sticking" of βNGF.[2] To check to see if the cytochrome c had some effect on the equilibrium

TABLE I

Equilibrium Constant for [$^{125}$I]βNGF Incubated For 24 and 48 Hours at Various Concentrations

24 Hours

| Counts of 100 µl Sample[a] | Concentration of [$^{125}$I]βNGF x $10^{-10}$M[b] | % cpm Dimer | % cpm Monomer | Keq ($10^{-11}$M) |
|---|---|---|---|---|
| 6837 cpm | 24.4 | 94% | 6% | 3.7 |
| 5633 cpm | 20.1 | 92% | 8% | 5.5 |
| 4845 cpm | 17.3 | 94% | 6% | 2.7 |
| 2152 cpm | 7.7 | 88% | 12% | 4.8 |
| 1427 cpm | 5.1 | 90% | 10% | 2.3 |
| 1045 cpm | 3.7 | 87% | 13% | 2.9 |
| 520 cpm | 1.9 | 83% | 17% | 2.6 |
| 328 cpm | 1.2 | 85% | 15% | 1.3 |
| | | | | Av.: $3.2 \pm 1.4 \times 10^{-11}$ |

48 Hours

| | | | | |
|---|---|---|---|---|
| 9800 cpm | 34.9 | 97% | 3% | 1.3 |
| 6560 cpm | 23.4 | 96% | 4% | 1.6 |
| 5645 cpm | 20.1 | 92% | 8% | 5.5 |
| 2943 cpm | 10.5 | 92% | 8% | 3.0 |
| 2484 cpm | 8.9 | 90% | 10% | 4.0 |
| 1549 cpm | 5.5 | 89% | 11% | 3.0 |
| 1309 cpm | 4.7 | 87% | 13% | 3.5 |
| 624 cpm | 2.2 | 90% | 10% | 0.97 |
| 474 cpm | 1.7 | 90% | 10% | 0.77 |
| | | | | Av.: $2.6 \pm 1.6 \times 10^{-11}$ |

Av. of 24 and 48 hour Keq: $2.9 \pm 1.5 \times 10^{-11}$

[a]Counts (minus background) per 100 µl of incubation medium separate from that used for electrophoresis.

[b]Determined by dividing counts in 100 µl sample by 1058 cpm/ng [$^{125}$I]βNGF.

constant, the equilibrium constant was determined without the added cytochrome c. This equilibrium constant was approximately $3 \times 10^{-11}$M; however, there was much more "sticking" (60-90% of the protein compared to less than 1%)[2] in the absence of the cytochrome c than in its presence. Therefore, all the equilibrium constants were determined in the presence of 2.5 mg/ml cytochrome c.

Hedrick and Smith (23) have demonstrated that it is possible to separate

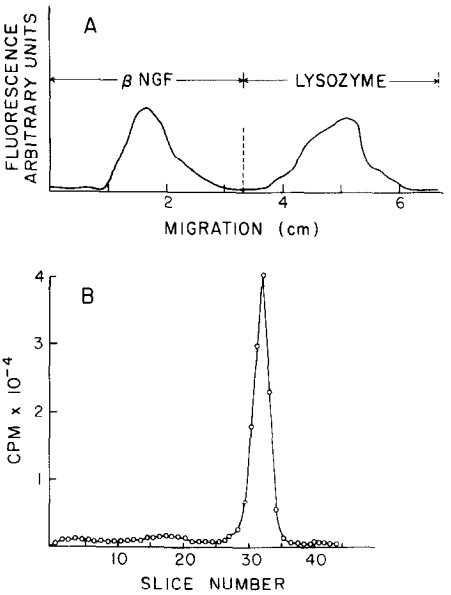


Figure 1: A. 100 µg of lysozyme and 100 µg of βNGF were analyzed on 7½% polyacrylamide gels (.5 x 8 cm) as described in "Materials and Methods". Migration was from left to right. The dotted line indicates the point at which the gels were cut to separate the βNGF monomer and dimer and arrows indicate top (βNGF) and bottom (lysozyme) portions of the gel.

B. 164 ng of [$^{125}$I]βNGF were incubated at room temperature in the presence of SDS for 20 hours. The protein was then analyzed on 12% SDS gels (.5 x 8 cm) as described in "Materials and Methods". Migration was from left to right. The single peak migrates as the βNGF monomer.

monomers, dimers, and trimers, etc. using polyacrylamide gel electrophoresis. To see if it would be possible to separate the monomer and dimer of βNGF, a mixture of βNGF and lysozyme were analyzed on 7.5% polyacrylamide gels (Fig. 1A). Lysozyme was used as a model for the monomer of βNGF since its isoelectric point (approximately 11) and molecular weight (14,100) are similar to the isoelectric point and molecular weight of the monomer of βNGF. As can be seen (Fig. 1A), lysozyme and βNGF are completely separated on this gel system. The gels containing the [$^{125}$I]βNGF were sliced in a spot corresponding to the point between the lysozyme and βNGF (Fig. 1A). The top and bottom portions of the gels (Fig. 1A) were placed in tubes and counted as described in "Materials and Methods".

It was also of interest to determine if the [$^{125}$I] iodide was bound to intact βNGF. Even though there was almost 100% TCA precipitable counts, there may have been some cleavage during the iodination procedure which would still be TCA precipitable. In Fig. 1B, it can be seen that on SDS-polyacrylamide gels all the radioactivity added to the gels is in one peak corresponding to the monomer of βNGF.

## DISCUSSION

We have shown that [$^{125}$I]βNGF has an equilibrium dissociation constant of approximately 3 x $10^{-11}$M. This dissociation constant is almost 4 orders of magnitude smaller than that reported for 2.5S NGF (17). If the constant obtained by Young, et al. for 2.5S NGF is correct, the receptor studies along with any other studies obtained using iodinated βNGF may be in error. However, without cytochrome c present, approximately 90% of the NGF can "stick" to glass, polyethylene, etc.[2] and this may be an explanation for the difference in the results reported here and those reported by Young, et al. (17). In our studies, since we can determine the number of counts per ng of βNGF, it is possible to determine the actual concentration of [$^{125}$I]βNGF from the counts remaining in solution; thus, it is possible to circumvent the problem of "sticking".

If the constant obtained for [$^{125}$I]βNGF is also the same constant for native βNGF, which seems reasonable since one would not expect iodination to shift the equilibrium constant by any great degree, then at physiological concentrations; such as 10 ng/ml, βNGF is approximately 100% dimer. It should be pointed out that Moore and Shooter (24) derived an equilibrium constant from their studies on the formation of $\beta^2$NGF from $\beta^1$NGF and $\beta^3$NGF. The constant they obtained (3 x $10^{-10}$M) was for a solution at 4°C and pH 4.0; they suggested that this would give an equilibrium constant of approximately 3 x $10^{-11}$M at 37°C and neutral pH (24). This derived constant is in very excellent agreement with that found in this study.

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