center in the DMF-modified ferredoxin by the dithiol, an alteration in the redox properties of the reacting species must have occurred since reduction does not occur in aqueous solution. Whether this change is due to incorporation of the dithiol molecules into micelles, to partial denaturation of the protein, or to a combination of the two effects still has to be clarified. These observations suggest that some dithiols will not be useful for core extrustion in aqueous Triton/DMF.

At a starting pH range of 8.0–8.5 and a Fd concentration near 0.1 mM, optimal ranges for quantitative core extrusion from the active site of spinach Fd by PhSH can be summarized as 10–30% (v/v) DMF, 5–6% (v/v) Triton, and 50–100 mM PhSH, ≲50 mM ionic strength. Very recently, we have obtained quantitative core extrusion of the two [4Fe–4S] centers in *C. pasteurianium* Fd under these conditions (D. M. Kurtz, Jr., unpublished results). To our knowledge the studies reported here represent the first detailed descriptions of the kinetics and equilibria of core extrusion reactions as well as the nature of each species participating in these reactions. We think that such descriptions for simple ferredoxins can serve as guides to choosing optimal conditions for selective core extrusion and site identification in as yet incompletely characterized complex iron–sulfur proteins.

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Tryptophan Residues of the γ Subunit of 7S Nerve Growth Factor: Intrinsic Fluorescence, Solute Quenching, and *N*-Bromosuccinimide Oxidation†

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ABSTRACT: The environment of tryptophan residues of the γ subunit derived from the 7S nerve growth factor has been studied by intrinsic fluorescence, solute quenching, and oxidation with *N*-bromosuccinimide (NBS). The native protein has a fluorescence emission maximum of 345 nm with a tryptophan quantum yield of 0.10. A red shift in the emission maximum occurs between 3 and 6 M urea; a 20% increase in quantum yield occurred at 7–8 M urea. NBS (20–24 mol of NBS/mol of protein) completely oxidized one tryptophan of the γ subunit, causing a 70% quenching of the intrinsic fluorescence, a complete loss of esterolytic activity toward synthetic substrates, an inability to combine with α and β subunits to re-form the 7S complex, and a shift of about 12% of the structure from β strands to random coil as determined by circular dichroism. About 80% of the fluorescing tryptophans are accessible to quenching by acrylamide but not to potassium iodide. These results suggest the presence of an exposed tryptophan contributing ≥70% of the native fluorescence, located near a negative charge, critical to the esterase activity and possibly to interaction with the β subunit to re-form 7S NGF.

Nerve growth factor (NGF)[1] as isolated from the mouse submaxillary gland is a 7S oligomer (M_r 130 000–140 000). The complex consists of three different subunits, α, β, and γ, in the stoichiometric ratio of 2:1:2 ($\alpha_2\beta\gamma_2$). The oligomer can be dissociated to the free subunits under a variety of conditions (Varon et al., 1968; Au & Dunn, 1977; Palmer & Neet, 1980b).

The subunits apparently have different functions. The β subunit, itself a homo dimer, is responsible for eliciting neurite

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[1] Abbreviations: BAPNA, N^{α}-benzoyl-DL-arginine-*p*-nitroanilide; EDTA, disodium salt of ethylenediaminetetraacetic acid; NGF, nerve growth factor; NBS, *N*-bromosuccinimide; TAME, tosylarginine methyl ester; Tris, tris(hydroxymethyl)aminomethane.

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outgrowth. No known biological function has yet been attributed to the α subunit. In conjunction with the γ subunit it probably protects the β subunit from proteolytic attack in the 7S oligomer. The γ subunit is an arginine-specific protease which has been proposed to cleave the pro β-NGF to yield the βNGF in vivo (Angeletti & Bradshaw, 1971; Berger & Shooter, 1977). Other functions have also been suggested (Greene et al., 1971).

The selective alteration of specific functional groups can provide valuable information on the structure–function relationship of biologically active molecules. To this extent, the role of tryptophan residues in the biological activity of βNGF has been examined (Angeletti, 1970; Frazier et al., 1973; Cohen et al., 1980). No such information is available for the γ subunit of 7S NGF. In this report we describe the effect of NBS oxidation of the tryptophan residues of the γ subunit on the esterase activity and its ability to recombine with α and β subunits to form the 7S oligomer. We have also examined the environment of the tryptophans by intrinsic fluorescence, solute quenching with acrylamide and KI, and oxidation with NBS.

Materials and Methods

The 7S NGF was isolated in pure form from the submaxillary glands of Swiss Webster random bred mice (Charles River Breeders) by the method of Varon et al. (1967) with the modification of Stach et al. (1976). The γ subunit was purified from the 7S NGF by the method of Smith et al. (1968). The concentration of γ subunit was determined by absorbance at 280 nm by using the value of 1.56 for the extinction coefficient ($E_{280}^{0.1\%}$) (Smith, 1969).

Esterase Activity. The assay for esterase activity utilized either tosylarginine methyl ester (TAME) or N^{α}-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA) as substrate. With TAME, assays were monitored at 247 nm on a Gilford Model 240 spectrophotometer. Assays were done at 25 °C in 1 mL of 0.05 M phosphate buffer, pH 6.8, containing 0.5 mM TAME. The reaction was started by addition of the enzyme solution (5–10 μL) to give a final enzyme concentration of 9–10 μg/mL. With BAPNA assays were done in 0.05 M Tris buffer, pH 7.40, and the rate of *p*-nitroaniline production was monitored at 410 nm. When the esterase activity in the 7S oligomer was assayed for, the protein (35 μg/mL) was preincubated in 1 mL of 0.05 M phosphate buffer, pH 6.8, containing 1 M NaCl and 1 mM EDTA for 3 min, and the reaction was initiated by the addition of TAME (0.5 mM).

Tryptophan Estimation. Tryptophan was estimated by the NBS oxidation method of Spande & Witkop (1967) and the spectroscopic method of Edelhoch (1967). In the former 1 mL of protein having an absorbance of 0.5 at 280 nm was titrated with 2.5-μL aliquots of *N*-bromosuccinimide (NBS) (aqueous stock solution of 4.5 mM) until the decreasing absorbance at 280 nm reached a constant value. Tryptophan content was then calculated by using the appropriate equation (Spande & Witkop, 1967).

Fluorescence Measurements. Fluorescence measurements were made in a 1-cm cuvette at 20 °C with an Aminco-Bowman spectrofluorophotometer equipped with elliptical condensing lens, a xenon arc lamp, and a Ratio II accessory. The excitation band width was 2 nm. All measurements were made in the range where emission was linear with protein concentration. Solutions having absorbances not exceeding 0.1 at the excitation wavelength were used. The relative quantum yields of tryptophan fluorescence was calculated according to eq 1 where ϕ is the quantum yield, A the area under the emission spectrum, and Abs the absorbance of the solution at the excitation wavelength (295 nm). The standard was an aqueous solution of *N*-acetyltryptophanamide with a known quantum yield of 0.14 (Eisinger, 1969).

$$\frac{\phi(\text{protein})}{\phi(\text{standard})} = \left[\frac{A(\text{protein})}{A(\text{standard})}\right]\left[\frac{\text{Abs(standard)}}{\text{Abs(protein)}}\right] \quad (1)$$

Fluorescence Quenching. Quenching of protein fluorescence with acrylamide was followed by progressive addition of small aliquots of a 6 M acrylamide solution. An excitation wavelength of 295 nm was used to ensure that the exciting light was absorbed almost entirely by tryptophan residues. However, since acrylamide has a molar extinction coefficient of 0.23 at 295 nm (Parker, 1968), fluorescence intensities were corrected for self-absorption of incident light by using the relationship of McClure & Edelman (1967).

Quenching with KI was done in a similar manner by using a stock solution of 6 M KI containing 0.1 mM sodium thiosulfate to prevent free iodine formation. For elimination of the effect of ionic strength, a similar titration was also done with a stock solution of 6 M NaCl.

The fluorescence quenching was analyzed by the modified Stern–Volmer plot for multitryptophan containing proteins according to eq 2 (Lehrer, 1971), where F_0 is the fluorescence

$$\frac{F_0}{\Delta F} = \frac{1}{f_a} + \frac{1}{f_a K_q[\phi]} \quad (2)$$

intensity at the emission maximum in the absence of quencher and ΔF is the change in intensity in the presence of quencher. ϕ is the quencher concentration. A plot of F_0/F vs. $1/Q$ will yield a straight line of slope $(f_a K_q)^{-1}$ and intercept $1/f_a$ with K_q = intercept/slope. f_a represents the fraction of tryptophan residues which are accessible to the quencher, and K_q is the Stern–Volmer quenching constant.

Circular Dichroism (CD) Measurements. Spectra were recorded at 25 °C with a JASCO-J40 spectropolarimeter having automatic slit control, using 0.1- and 1-cm path length cuvettes. The reported spectrum is the average of two separate protein samples each of which was recorded 2 times. Protein solutions with concentrations of 0.078 and 0.506 mg/mL were used in far-UV (240–190 nm) and near-UV (300–250 nm) measurements, respectively. Spectral band width was maintained at 1 nm. Mean residue ellipticity values were calculated by using a mean residue weight of 111.15 for the γ subunit.

Gel Filtration. A column of Sephadex G-75 of 30-mL bed volume was equilibrated with 0.05 M phosphate buffer, pH 6.8, containing 30 μM Zn^{2+}. One milliliter of protein solution in the same buffer containing about 900 μg of protein was loaded on the column which was then eluted with the same buffer at a flow rate of 10 mL/h; 1-mL fractions were collected, and the absorbance at 280 nm was read.

Gel Electrophoresis. Slab gel electrophoresis was done under nondissociating conditions (Varon et al., 1967) to detect the 7S oligomer. Gel was stained with Coomassie Blue R 250.

Ultracentrifugation. Sedimentation velocity experiments were done in a Beckman Model E ultracentrifuge equipped with a photoelectric scanner. Runs were performed at 20 °C and 60 000 rpm with an An D rotor in which the sample cell was scanned at 280 nm. The $s_{20,w}$ for each protein was calculated from the rate of movement of the midpoint of each boundary (Schachman, 1959). Unless otherwise mentioned all experiments were done in 0.05 M phosphate buffer at pH 6.8.

Results

Fluorescence Studies. Native γ exhibits a peak at 345 nm when excited at 280 nm (excitation maximum wavelength)

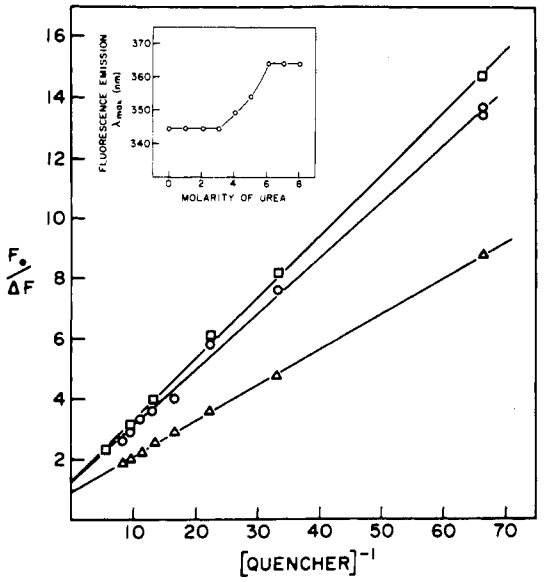

FIGURE 1: Modified Stern–Volmer plot of γ-subunit fluorescence quenching in 0.05 M phosphate buffer at pH 6.8. (○) γ subunit titrated with acrylamide; (△) 8 M denatured γ subunit titrated with acrylamide; (□) 8 M urea denatured γ subunit titrated with KI. (Inset) Fluorescence emission maximum of γ subunit as a function of urea concentration.

or at 295 nm. Excitation at 295 nm ensures that all the light is absorbed by tryptophan since the extinction coefficient of tryptophan at this wavelength is considerably higher than that of tyrosine. Tyrosine did not contribute to the fluorescence emission as calculated by the method of Longworth (1971).

The fluorescence emission maxima and intensity of proteins are very sensitive to changes in solvent polarity, and the change in λ_{max} can be used to monitor changes in the microenvironment of the tryptophan residues as the protein is denatured. The inset to Figure 1 shows the change in emission maximum as a function of urea concentration. A red shift in emission maximum is seen only above 3 M urea and attains a constant value (360–364 nm) at 6–8 M urea. The quantum yield of tryptophan fluorescence also increases from a value of 0.10 for the native to 0.12 for the protein in 8 M urea. This represents an approximately 20% increase in quantum yield. The observed changes in fluorescence properties in urea are due to the exposure of the buried tryptophans to the solvent. These residues may have a low quantum yield and not contribute much to the fluorescence. If, then, the fluorescence of native γ is mostly due to the "exposed" tryptophans, an estimation of the degree of exposure would facilitate a more straightforward interpretation. A useful method for such a measurement is the solute (as opposed to solvent) perturbation technique (Lehrer, 1971). The basis of this method is that certain low molecular weight substances known as "quenchers" are able to quench tryptophanyl fluorescence by a collisional mechanism, i.e., by physical contact with the indole ring of the excited tryptophan. The susceptibility of a residue to such a collisional quenching would depend on the degree of its exposure and potential ionic interactions. For example, an anionic quencher, such as I^- is much more likely to quench a tryptophan surrounded by positively charged residues and less likely to quench one in a negatively charged region owing to repulsion (Lehrer, 1971). Acrylamide is a neutral molecule and a very efficient quencher since it does not discriminate between residues in different charged environments (Eftink & Ghiron, 1976).

We have used acrylamide and potassium iodide in our quenching studies and analyzed the data by the modified Stern–Volmer plot (Figure 1) which assumes that all accessible fluorophors have the same quenching constant. The value of f_a which represents the fraction of tryptophan residues accessible to the quencher was obtained from the y intercept. Table I summarizes the quenching parameters for γ subunit which has six tryptophan and nine tyrosine residues (Thomas et al., 1981a). With acrylamide 81% or five residues are apparently accessible to the quencher; 100% accessibility is obtained in 8 M urea. As expected there is an increase in the value of the Stern–Volmer quenching constant, K_q, which is a measure of the ease with which residues are quenched. Titration of native γ subunit with KI gave about 13% quenching at a final I^- concentration of 0.12 M (data not shown). However, NaCl at the same concentration gave about 10% quenching. Thus, this quenching is attributed simply to the ionic strength effect with little or no specific quenching with KI, suggesting that the tryptophans are in a negative environment (Lehner, 1971). After denaturation in 8 M urea, however, 82% of the tryptophans are accessible (Figure 1). NaCl does not quench the fluorescence under these conditions. Unfolding of the molecule in 8 M urea probably removes the negative charges from the proximity of the tryptophan(s). Even under these conditions, however, there is still about one mean residue which cannot be quenched with I^-.

Table I: Fluorescence Quenching Parameters

protein	quencher	K_{SV} (M^{-1})	f_a	% sites accessible
native γ	acrylamide	6.7	0.8	81 (4.9) [a]
γ + 8 M urea	acrylamide	8.0	1.0	100 (6.0)
native γ	KI	0	0	none [b]
γ + 8 M urea	KI	5.9	0.8	82 (4.9)

[a] The numbers in parentheses indicate the average number of tryptophan residues if one assumes that all six tryptophans contribute equally to the fluorescence (see Discussion). [b] Corrected for NaCl control; see text.

Reaction with N-Bromosuccinimide. Oxidation with NBS offers another method of estimating exposed tryptophan residues (Spande et al., 1966). Although the pH optimum of the reaction is 4, it can still be carried out at higher pH values; however, some side reactions occur at the same time. We have used this method to determine the number of exposed tryptophan residues in the native and urea-denatured protein. The buffer solutions used were 0.05 M sodium acetate, pH 4.0, and 0.05 M sodium phosphate buffer, pH 6.8 (Figure 2). In the native protein at pH 6.8 only one tryptophan is oxidized at an NBS:protein molar ratio of approximately 20:1. This ratio has been found to vary from 20 to 24 mol of NBS/mol of protein for different batches of γ subunit; however, an average of one tryptophan is consistently oxidized. After denaturation in 8 M urea, 3.5–3.8 residues are oxidized at an NBS:protein molar ratio of approximately 16:1. The oxidation of the first tryptophan residue occurs at a considerably lower molar ratio than that obtained in the absence of urea at pH 6.8. Even at pH 4.0, the optimum pH for the specific oxidation of tryptophan residues, only 1.8–2.0 residues are oxidized, although the reactivity of the tryptophans is greater than that at pH 6.8. With the incorporation of urea at pH 4.0, however, approximately 5.6 tryptophan residues are now oxidizable. The reported tryptophan content for the γ subunit is six residues (Thomas et al., 1981a) by the spectroscopic method of Edelhoch (1967). We obtained a value of 5.6 residues by the same method. Apparently, all the tryptophan residues are accessible to NBS only after the molecule has been completely denatured in 8 M urea at pH 4.0. Apparently, even in 8 M urea at pH 6.8 the molecule has residual structure, perhaps

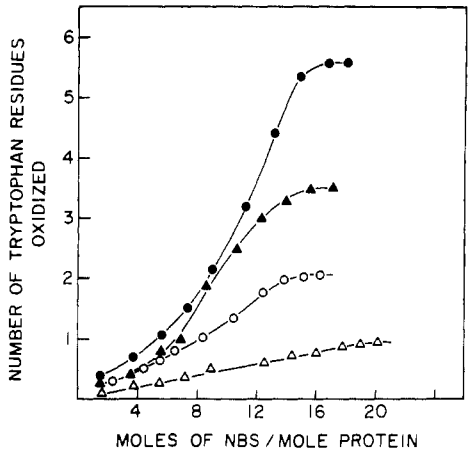

FIGURE 2: Reaction of tryptophan residues of γ subunit with *N*-bromosuccinimide. Enzyme concentration was 11 μM. Increments of NBS were added as described under Materials and Methods. The figure represents results obtained with one batch of γ subunit. (△) In 0.05 M phosphate buffer at pH 6.8; (▲) in 0.05 M phosphate buffer at 6.8 plus 8 M urea; (○) in 0.05 M acetate buffer at pH 4.0; (●) in 0.05 M acetate buffer at pH 4.0 plus 8 M urea.

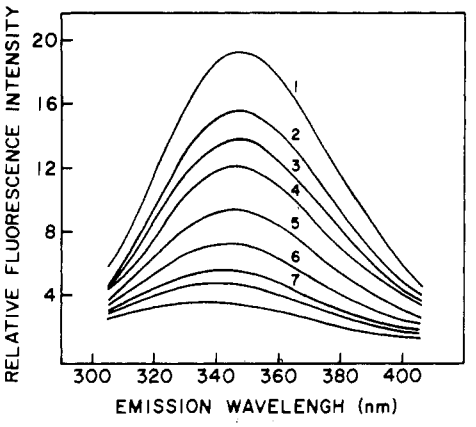

FIGURE 3: Quenching of γ-subunit fluorescence by oxidation with NBS. 2.5-μL aliquots of NBS (4.5 mM stock solution) were added to 1 mL of enzyme solution (3.6 μM) in 0.05 M phosphate buffer, pH 6.8, and the spectra recorded at each addition after excitation at 295 nm. Percent quenching was calculated by comparing the area under the peaks. (1) Native protein; (2) 0.15 tryptophan oxidized; (3) 0.30 tryptophan oxidized; (4) 0.60 tryptophan oxidized; (5) 0.75 tryptophan oxidized; (6) 0.90 tryptophan oxidized; (7) 1.04 tryptophans oxidized.

attributed to the five disulfide bonds, in which the remaining tryptophans may be partly buried, and therefore only about 3.5 residues are oxidized. Alternatively, the NBS may be sterically hindered from reaction with the remaining residues.

Of interest at this point was the effect of NBS on the fluorescence properties of the γ subunit (Figure 3). Progressive addition of NBS causes quenching, and when one tryptophan is oxidized, 70% of the fluorescence is quenched. Also, there is a 5-nm blue shift in the λ_{max} of emission.

NBS oxidation had a profound effect on the activity of γ. Activity progressively decreased, and at an NBS:protein molar ratio of 20:1 the γ subunit had no esterase activity toward TAME (Figure 4) or toward BAPNA (data not shown). Increasing substrate concentration up to 2.5 mM did not elicit any activity from the oxidized protein. Since the K_m for the native enzyme with TAME as substrate is approximately 0.15 mM, the loss in activity is most likely due to a complete loss in catalytic activity and not simply an increase in K_m of $\geq 10^3$ for oxidized γ. Incubation of the enzyme with TAME at a concentration of 4–8 mM concomitant to treatment with NBS did not protect the enzyme. Although NBS is considered to

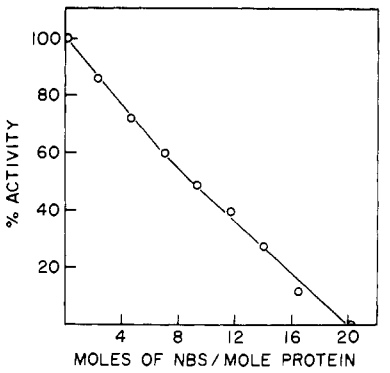

FIGURE 4: γ esterase activity as a function of molar excess of NBS. Enzyme concentration was 36 μM.

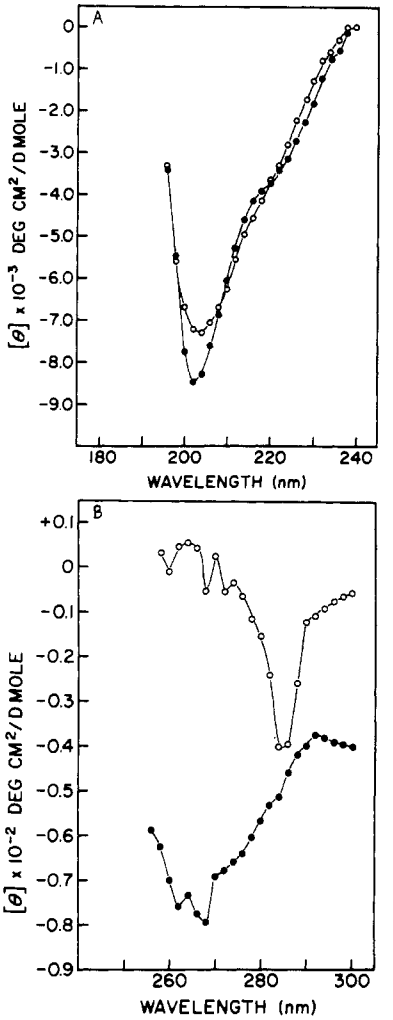

FIGURE 5: CD spectra of native (○) and one tryptophan oxidized γ subunit (●) in 0.05 M phosphate buffer at pH 6.8. (A) Far-UV spectrum (protein concentration 0.078 mg/mL); (B) near-UV spectrum (protein concentration 0.506 mg/mL).

be selective for tryptophan residues, other functional groups are affected, especially at pH values higher than 4 (Ramachandran & Witkop, 1959; Schmir & Cohen, 1961; Ramachandran, 1962; Frazier et al., 1973). The biphasic nature of the curve in Figure 4 may therefore be indicative of the simultaneous oxidation of some other residues.

CD spectra of the native and oxidized γ subunit were recorded in the far-UV (Figure 5A) and near-UV (Figure 5B) to determine if the oxidation of one tryptophan had any effect on the secondary structure of γ. In the far-UV, overall features of the two spectra were similar but not identical. Both the

Table II: Estimated Fractions of α Helix, β Structure, and Random Coil of Native and Oxidized γ Subunit

	α helix		β sheet		random	
	native γ	oxidized γ	native γ	oxidized γ	native γ	oxidized γ
CD	0.10–0.13	0.10–0.13	0.27–0.30	0.15–0.17	0.48–0.52	0.61–0.64
predicted [a]	0.15		0.39		0.46	

[a] Predicted by the Chou–Fasman procedure. The random structure includes β turns.

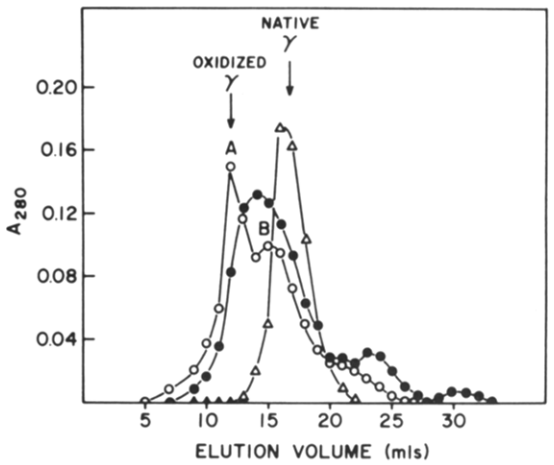

FIGURE 6: Gel filtration of a mixture of α, β, and γ subunit on Sephadex G-75 equilibrated with 0.05 M phosphate buffer, pH 6.8, containing 30 μM Zn^{2+}. (○) Control mixture of 300 μg of each subunit dialyzed for 24 h against above buffer; (●) experimental mixture consisting of the same quantity of α, β, and oxidized γ subunit dialyzed as above; (△) α subunit dialyzed as above. The arrows indicate the elution positions of native and oxidized γ subunit. The oxidized γ subunit also showed a slight shoulder at the elution position of native γ.

native protein and oxidized protein showed a minimum at 202–204 nm with a shoulder at 218 nm. The mean residue ellipticity value at the minimum was 18% greater for the oxidized as compared to the native protein. In the near-UV the native protein showed a minimum at 284 nm. Upon oxidation the trough at 284 nm was abolished, and new minima appeared at 262 and 268 nm with slight shoulders at 276 and 284 nm. From model compound studies (Horwitz et al., 1969, 1970; Holladay & Puett, 1976) the minimum at 284 nm is attributable to tyrosine transitions and the minimum at 262 and 268 nm to phenylalanine transitions. Suprisingly, the native protein did not show any positive peaks due to tryptophans in the 290–300-nm region.

Protein secondary structure fractions were estimated from the CD data by the method of Chen et al. (1974) (Table II). Evidently, the γ subunit is a protein possessing little α helix and mostly β and random structure. Trypsin, a serine protease, with which the γ subunit shares much sequence homology (Thomas et al., 1981a) has also been described as a nonhelical protein with mostly random structure (Jergensons, 1970). We have predicted the secondary structure of the γ subunit by the Chou–Fasman prediction method (1974). The structure agrees to a large extent with that reported by Thomas et al. (1981a) based on the sequence similarity of γ subunit with other serine proteases. The secondary structure fractions calculated from the predicted structure are in good agreement with values obtained from circular dichroism (Table II).

The effect of oxidation of one tryptophan residue of γ on its ability to form 7S NGF was also investigated by recombination experiments with α and β subunits. The subunits were

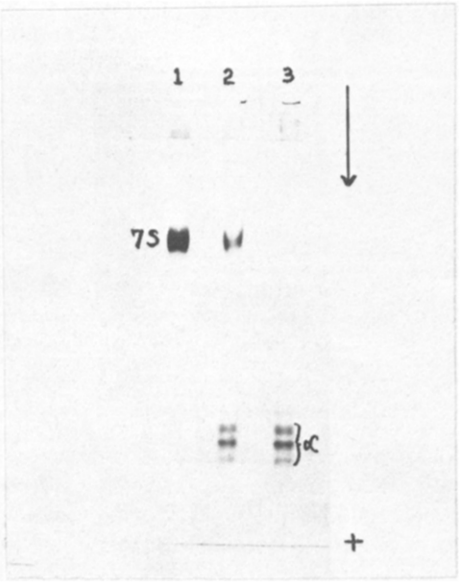

FIGURE 7: Polyacrylamide slab gel electrophoresis under conditions favoring 7S formation (Varon et al., 1967). Staining was with Coomassie Blue. (Lane 1) Native 7 S (15 μg); (lane 2) control mixture of α + β + γ (15 μg); (lane 3) experimental mixture of oxidized γ + β + α (15 μg).

Table III: Subunit Reassociation

subunits [a]	$s_{20,w}$	$s_{20,w}$ values obtained by Palmer & Neet (1980b)
α + β + γ	7.54, 4.29	6.74, 3.84 (without Zn^{2+})
α + β + γ (oxidized)	4.36	
β + γ	6.10, 2.27	5.84, 2.44 (with 50 μM Zn^{2+})
β + γ (oxidized)	3.80	
γ	2.25	
γ (oxidized)	3.65	
β	2.20	

[a] Mixtures of subunits (200 μg/mL each) were dialyzed overnight at 4 °C against 0.05 M phosphate buffer at pH 6.8 containing 30 μM $ZnSO_4$. Free γ and oxidized γ subunit (300 μg/mL each) and free β subunit (200 μg/mL) were dialyzed as above.

dialyzed against pH 6.8 buffer to facilitate 7S formation and then subjected to gel filtration at the same pH (Figure 6). The control mixture of α, β, and γ subunits showed a fraction eluting in the void volume at 12.5 mL (peak A) and a second fraction eluting at 14.5 mL (peak B). The mixture containing oxidized γ (experimental) showed only one peak at 14.5 mL (peak B). Free α and γ subunits eluted at ~17 mL. Native 7 S also eluted at the same position as peak A. However, γ subunit with one tryptophan oxidized eluted in the void volume. Before gel filtration, the control and experimental mixtures were also examined by gel electrophoresis under conditions favoring 7S formation (Figure 7). The native 7 S showed a single band (lane 1). The control mixture of α, β, and γ subunits showed a band corresponding to 7 S and bands due to α subunit (lane 2). In the experimental mixture of oxidized γ, α, and β there was no evidence of 7S formation (lane 3). Surprisingly, the γ subunit, either in the control or oxidized form, did not appear to be stained.

Subunit reassociation was also examined by sedimentation velocity experiments (Table III). The control mixture of α, β, and γ showed two species sedimenting with $s_{20,w}$ values of 7.54 S (corresponding to native oligomer) and 4.29 S (intermediate complex). With the mixture containing tryptophan oxidized γ subunit, there was only one sedimenting species with $s_{20,w}$ of 4.36 S, and there was no evidence of any 7S formation. Native γ sedimented with an $s_{20,w}$ of 2.25 S while the oxidized

Table IV: Oxidation of 7S Oligomer with NBS

NBS:7S [a] molar ratio	tryptophans oxidized	sp act. of γ subunit [b]	% act. of control
0	0	18.37	100
30-35	1	18.23	100
65-70	3	10.88	59
100-105	4.5-5.0	3.67	20
138-145	5.5-6.0	0	0
170-180	6.0-7.0	0	0

[a] Based on a molecular weight of 131 000 for the 7S complex, which contains 2 mol of γ subunit/mol of 7S. [b] Micromoles per minute per milligram of protein. Assayed after incubation with NaCl and EDTA (Palmer & Neet, 1980a).

material moved faster with an $s_{20,w}$ value of 3.65 S (consistent with the observation that the γ subunit aggregates after oxidation with NBS). A mixture of β and γ gave two species with $s_{20,w}$ values of 6.10 S and 2.27 S, and interestingly, β and oxidized γ gave a single species with an $s_{20,w}$ of 3.80 S.

The γ subunit has been postulated to function by proteolytically cleaving a precursor pro NGF dimer to the NGF dimer and, unlike a typical enzyme–product complex which rapidly dissociates, remain associated as a complex with the βNGF dimer (Angeletti & Bradshaw, 1971). The esterase activity of the γ subunit is suppressed in the 7S oligomer (Pattison & Dunn, 1975; Palmer & Neet, 1980a) or in combination with the βNGF (Bothwell & Shooter, 1978). Hence, it has been contended that the active site of the γ subunit is involved in the binding site for the βNGF. We have shown here that the γ subunit with one tryptophan oxidized does not readily form the 7S species. These results are consistent with the possibility that the essential tryptophan is in the binding site for the β on the γ subunit. If this is the case, the essential tryptophan should be protected from oxidation in the 7S oligomer. We have investigated this possibility by NBS oxidation of the 7S oligomer and assaying the esterase activity of the γ subunit. The result is summarized in Table IV. The γ subunit retains complete activity even when one tryptophan in the oligomer is oxidized and activity decreases as more tryptophans are oxidized.

Discussion

The fluorescence emission maximum of the native γNGF is at 345 nm. Teale (1960) first observed that the λ_{max} of fluorescence emission of denatured proteins is close to that of the free amino acid in water. Following this observation the position of emission maximum of a protein has been widely used as an index of "exposure" of tryptophan residues. The closer the λ_{max} of the protein to that of the free amino acid the more "exposed" the tryptophan(s) is (are) considered to be. However, such a correlation is only empirical (Kronman & Holmes, 1971). Various factors such as the presence of water molecules within the protein or interactions of the indole ring with moieties on the protein will influence the position of λ_{max}. Thus, the observed λ_{max} of emission of native γ subunit only reflects subtle environmental influences of the tryptophan in the protein and does not directly indicate the degree of its exposure.

Fluorescence quenching is a convenient technique for probing tryptophans in proteins. Unlike solvent perturbation techniques, the quencher in solute perturbation does not have to be in constant contact with the fluorophor. Fluorescence quenching also enjoys a distinct advantage over chemical modification studies in that the protein molecule is not permanently damaged.

The fluorescence quenching data obtained with acrylamide and the experiments with NBS at first appear to be contradictory. Acrylamide quenching results suggest that 80% of the tryptophan fluorescence is accessible to the quencher. NBS oxidation shows that there is only one tryptophan oxidizable at pH 6.8. This apparent contradiction can be explained as follows. Of the six tryptophan residues there is, in fact, only one on the surface as determined by NBS oxidation. This residue is largely responsible for the fluorescence and has a quantum yield of 0.10. When this residue is completely oxidized with NBS, 70% of the fluorescence is quenched (Figure 3), further supporting the contention that one tryptophan contributes predominantly to the fluorescence. Therefore, the 80% accessibility with acrylamide actually refers to the quenching of the single major fluorescent residue. On the other hand, more residues may, in fact, be accessible to acrylamide but have negligible fluorescence; the quenching observed will be still due to the highly fluorescent tryptophan. When the exposed tryptophan is completely oxidized, the fluorescence is due entirely to the buried tryptophans (Figure 3). This conclusion is supported by the fact that there is also a blue shift of 5 nm in the λ_{max}, since such a blue shift with quenching has been observed with most proteins and represents the emission maximum of the buried residues.

The γ subunit is a heterogeneous mixture of six different forms (Stach et al., 1976; Burton & Shooter, 1981; Thomas et al., 1981b), and it may be argued that the observed effects of NBS and acrylamide are restricted to some of these forms rather than all six. The fact remains that there is still only one tryptophan accounting for 70% of the fluorescence. We have noticed no difference in fluorescence of different preparations of γ containing different proportions of γ_1, γ_2, and γ_3.

Titration of native γ subunit with KI gave little or no specific quenching due to iodide. Lehrer (1971) has shown that quenching with I^- is markedly influenced by charged residues about the tryptophan such that negative charges cause low quenching owing to repulsion of like charges. On this basis our results would suggest that the single tryptophan on the surface is in a negatively charged region and is therefore not quenched to any appreciable extent; this is consistent with the decrease in fluorescence that occurs with decrease in pH. Following denaturation in 8 M urea an average of five residues is accessible and is probably located in a positively charged environment. One other tryptophan either is in a negatively charged region or is buried in a region of residual structure and is therefore inaccessible. Since acrylamide quenching in 8 M urea gives 100% accessibility, it seems likely that the inaccessible fraction remains in a negatively charged region.

These studies indicate that the buried tryptophans contribute very little to the fluorescence of the native protein. The low quantum yield of these tryptophans could be attributed to the influence of side chains in the folded protein. It is known from model compound studies that tryptophan fluorescence can be quenched by neighboring protonated acidic groups. Protonated histidine has been shown to quench the tryptophan fluorescence in a number of proteins (Shinidzky & Goldman, 1967) by the formation of a nonfluorescent charge-transfer complex. Quenching effect due to carboxylate groups has also been implicated in several proteins. However, the effect of amino acid sequence on the fluorescence is not yet clearly understood. Edelhoch et al. (1968) have shown that the peptide bond itself can quench fluorescence.

The γ subunit has nine tyrosine and six tryptophan residues, yet we failed to observe any tyrosine contribution in the ex-

citation or emission of fluorescence even in the denatured state or under conditions where the tryptophan fluorescence was eliminated, e.g., by NBS oxidation. In proteins containing both tryptophan and tyrosine, the fluorescence of the former is wlll-known to dominate, although some exceptions are reported. A number of factors are known to quench tyrosyl fluorescence in proteins also containing tryptophan residues (Longworth, 1971). Chief among these is energy transfer to the latter. For this to occur, certain distance requirements have to be satisfied, and once the molecule is completely denatured, it should be possible to isolate the tyrosine contribution. However, under our experimental conditions the molecule does not appear to be completely denatured even in 8 M urea (see Results). Disulfide bonds and polar side chains in the proximity can also quench tyrosine fluorescence. An examination of the amino acid sequence of γ (Thomas et al., 1981a) shows that tyrosine fluorescence could be quenched by any/all of these factors.

Our results clearly indicate that the oxidation of one tryptophan not only profoundly affects the properties of the γ subunit but also disturbs subunit interactions with α and/or β subunits. Sedimentation velocity experiments show that while the oxidized γ subunit cannot participate in 7S formation, it can still form an intermediate complex with the β subunit. This complex (3.80 S), however, differs from the native βγ complex (6.10 S) and probably has a different stoichiometric composition. It may be that the oxidized γ subunit can form a complex of the type γββ (M_r 50000) such that the binding of γ to one $\beta_{1/2}$ hinders the binding of a second molecule of γ to the other $\beta_{1/2}$. Such a complex could also arise from the dissociation of a molecule of γ from an initially formed unstable complex of the type γ–β–β–γ. Alternatively, the binding of the oxidized γ subunits to the β dimer could weaken the $\beta_{1/2}$–$\beta_{1/2}$ interaction and lead to an intermediate species of the type $\gamma\beta_{1/2}$(M_r 38000). Either type of complex could account for the observed 3.80S species. However, a nonspecific interaction between the oxidized γ and β subunits cannot be eliminated. The fact that the γ subunit after oxidation cannot interact to form 7 S but can still form a complex with the β subunit would implicate a tryptophan on the γ subunit controlling an essential conformational feature for 7S formation. Our CD data (Table II) show that the γ subunit goes from a predominantly unordered state (48–50% random) to a more unordered state (60–65% random) upon oxidation and that this change occurs at the expense of the β structure in the native molecule. Interestingly, none of the six tryptophans occur in the α-helical region of the Chou–Fasman predicted structure. Two residues (at positions 14 and 50) are apparently in β-sheet regions while the other four occur in β turns.

The data from NBS oxidation of the 7S molecule show that 100% esterase activity is retained after oxidation of one tryptophan (Table IV). This result is possible even if the tryptophan on the γ subunit is exposed in the 7 S and not protected by the binding of the β subunit at all; it may just be less reactive than some other tryptophan residue on the α or β subunit. Subtle subunit interactions in the 7 S could conceivably alter the reactivities of tryptophan(s) in individual subunits. This appears to be so at least in the case of the β subunit. Frazier et al. (1973) have reported an NBS:$\beta_{monomer}$ molar ratio of 3:1 at pH 4.0 for the oxidation of one tryptophan. Considerably higher ratios have to be reached for this to occur in the 7S oligomer (Table IV). Alternatively, the tryptophan on the γ subunit may be protected by the binding of the β subunit. The decrease in esterase activity as more tryptophans are oxidized could be attributed to a conformational change of the 7S molecule consequent to the oxidation of one residue, thereby exposing the tryptophan on the γ subunit.

The oxidation of one tryptophan on the γ subunit is accompanied by a loss in esterase activity and a shift of ~12% of the structure from β strands to random coil. Therefore, the loss in activity may be attributed to the specific oxidation of the tryptophan indole ring to the oxindole and/or conformational change of the molecule. Our results also indicate that the ability of oxidized γ subunit to interact with α and β subunits is significantly affected. While it can form a complex with the β subunit (albeit different from the native γβ complex), it seems unable to form the 7S oligomer. Although free α and γ subunits do not interact (Palmer & Neet, 1980b), it would appear that the oxidation of one tryptophan somehow alters the ability of the γβ complex to interact with α to form the 7S molecule. If, however, the binding site for the β on the γ subunit is lost on oxidation of the tryptophan (and a γβ complex is formed by nonspecific interaction), 7S formation will be precluded. This is consistent with the hypothesis that the active site of the γ-protease is required for interaction with the β subunit as an enzyme–product relationship (Berger & Shooter, 1977). However, an unambiguous interpretation does not seem to be possible at this stage.

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Nuclear Magnetic Resonance Studies of Xenon-129 with Myoglobin and Hemoglobin†

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ABSTRACT: Nuclear magnetic resonance studies of ^{129}Xe are consistent with one kinetically distinguishable binding environment in methemoglobin and two in metmyoglobin. The Xe binding site in methemoglobin is assigned to a cavity formed by the A–B and G–H corners of the globin chain [Schoenborn, B. P. (1965) *Nature (London) 208*, 760–762]. The small differences between α-hemoglobin and β-hemoglobin are not resolved by the NMR experiments. The Xe association rate constant at 18 °C with methemoglobin is greater than 6×10^7 M^{-1} s^{-1} with an activation barrier of approximately 13 kcal/mol. One of the binding sites in metmyoglobin is associated with a cavity on the proximal side of the porphyrin ring, opposite the O_2 binding site [Schoenborn, B. P., Watson, H. C., & Kendrew, J. C. (1965) *Nature (London) 207*, 28–30]. An estimate of the association rate constant of Xe at 18 °C is 1×10^7 M^{-1} s^{-1} with an activation barrier of approximately 16 kcal/mol. The second metmyoglobin binding site has similar NMR and kinetic properties of those for methemoglobin.

Myoglobin and hemoglobin bind xenon in specific relatively nonpolar cavities within the protein matrix. X-ray crystallographic studies reveal that sperm whale metmyoglobin binds xenon in the proximal cavity equidistant between the coordinated proximal histidine (F8) and a pyrrole group of the heme ring (Schoenborn et al., 1965). A second binding site has been reported in sperm whale alkaline myoglobin (pH 9.4) (Schoenborn, 1969). The location of this cavity is similar to the binding site found in horse hemoglobin—a cavity formed by the G–H and A–B helix corners close to the external surface (Schoenborn, 1965) (Figure 1). Equilibrium binding studies are consistent with a single association constant for Xe–hemoglobin complex on the order of 200 M^{-1} (Conn, 1961). The Xe–metmyoglobin interaction is better described with two association constants of approximately 190 M^{-1} and 10 M^{-1} (Ewing & Maestas, 1970). While these studies contain structural and thermodynamic information, they reveal little about the dynamics of Xe binding. Inspection of the protein crystal structures makes it clear that the Xe binding sites are inaccessible to ligand without cooperative protein motions to allow transient passage. The dynamics of Xe binding can be used to probe these protein motions. Kinetic studies have been made of the protein barriers associated with the binding of O_2 and CO to deoxymyoglobin by using optical techniques (Austin et al., 1975); however, Xe is an inert ligand that offers some unique features for probing barriers associated with a different class of binding sites, and its motion can be monitored directly by Xe NMR.

The use of NMR in studying chemical exchange is well established (Emsley et al., 1965). Kinetic information is contained in the transverse relaxation times and hence in the line shape of the resonance peak. Depending on the exchange rates and the chemical shifts, a process can be described as being in slow, intermediate, or fast exchange. In the limit of slow exchange a separate resonance is observed for each environment. For two sites this condition is met when $(\tau\Delta\omega)^2 \gg 1$ where τ is the lifetime of state and $\Delta\omega = 2\pi\Delta\nu$ with $\Delta\nu$

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