Modification of Staphylococcal Nuclease with Nitrophenylsulfenyl Halides*

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ABSTRACT: Staphylococcal nuclease was modified specifically at tryptophan residue 140 by the addition of a series of bulky nitrophenylsulfenyl moieties. Despite considerable disruption of the α-helical content estimated by circular dichroism, these modified nucleases retained full immunological reactivity, about 50% enzymatic activity against both DNA and RNA, and the capacity to be as fully protected by ligands from tryptic digestion as is the native enzyme. These studies support the view that tryptophan is not involved directly in the catalytic function of nuclease, but that the tryptophan side chain is accommodated tightly between two stretches of α helix, providing necessary structural stabilization.

Nitrophenylsulfenyl (NPS)[1] halides have been introduced as specific reagents for tryptophan and cysteine residues of proteins (Scoffone *et al.*, 1968; Fontana and Scoffone, 1971). We have used a series of these reagents in the selective modification of the single tryptophan residue 140 of staphylococcal nuclease, a single-chain polypeptide of 149 residues with no cysteine (Taniuchi *et al.*, 1967). In the recently completed molecular model of nuclease based upon X-ray diffraction analysis (Arnone *et al.*, 1969, 1971), the tryptophan residue is tightly accommodated between two major stretches of α helix. Oxidation of Trp^{140} previously with performic acid (Omenn *et al.*, 1970b) or with BNPS-skatole (Omenn *et al.*, 1970a) drastically disrupted the native conformation. The NPS moieties introduced do not greatly change the hydrophobic nature of the tryptophan side chain. The results of studies presented here have indicated the constraints with which the tryptophan is accommodated in the three-dimensional structure of nuclease.

Materials and Methods

2-Nitrophenylsulfenyl chloride (NPS-Cl) was purchased from Sigma. 2,4-Dinitrophenylsulfenyl chloride (DNPS-Cl) was made according to the method of Kharasch *et al.* (1950). 2-Nitro-4-carboxyphenylsulfenyl chloride (NCPS-Cl) (Veronese *et al.*, 1968) was a generous gift of Dr. A. Fontana, Institute of Organic Chemistry, University of Padova, Padua, Italy. Nuclease (Morávek *et al.*, 1969) and anti-nuclease antiserum (Omenn *et al.*, 1970c) were prepared as described previously. Physical, immunological, and enzymatic methods were those applied to the characterization of other nuclease derivatives (Omenn *et al.*, 1970a,b). Viscosity measurements were carried out at 20 ± 0.01° in an Oswald viscometer, using 0.01 M Tris-HCl buffer (pH 8.0) containing 0.1 M NaCl. The outflow times found with different concentrations of protein were referred to the outflow time of the buffer. For 1.0 ml of buffer, outflow time was 210 sec.

NPS Derivatives of N-Acetyl-L-tryptophan. By the method of Scoffone *et al.* (1968), *N*-acetyl-L-tryptophan (1.0 mmole) was treated with 1.2 mmoles of NPS-Cl, DNPS-Cl, or NCPS-Cl in glacial acetic acid at room temperature for 5 hr. The derivatives were purified by repeated precipitation with ether. The homogeneity of the derivatives was checked on thin-layer chromatography. Ultraviolet absorption spectra were obtained with a Cary 15 spectrophotometer, using solutions containing 20 μg of derivative/ml (Table I). Fluorescence spectra were obtained with an Aminco-Bowman spectrophotofluorometer, using dilute solutions.

NPS Derivatives of Nuclease. To a solution of 17 mg (1.0 μmole) of nuclease in 3 ml of 50% acetic acid, 10 μmoles of NPS-Cl in 0.2 ml of glacial acetic acid was added. After 3 hr, the protein was precipitated with 40 ml of acetone containing 2 ml of concentrated HCl at room temperature. The protein precipitates were centrifuged, dissolved in 1 ml of water, and reprecipitated twice in a similar manner. The final precipitates were dissolved in 3 ml of water and lyophilized. The recovery of modified nuclease was always more than 90%. DNPS-nuclease and NCPS-nuclease were prepared identically, with DNPS-Cl and NCPS-Cl as reagents, respectively.

Results

Completeness of Reaction. Calculations of moles of NPS introduced per mole of nuclease were based upon ultraviolet absorption spectra (Figure 1), with maxima at about 365 nm for the NPS-tryptophanyl moieties and at about 280 nm for the combined tyrosyl and NPS-tryptophanyl chromophores. The molar absorptivities for these maxima for each NPS-tryptophan derivative are given in Table I. Spectra of the modified nucleases were obtained both in 80% acetic acid and in water, pH 7. Nuclease concentration was determined independently by quantitative amino acid analysis after acid hydrolysis. For each of the three NPS derivatives, 1 mole of nitrophenysulfenyl chromophore was introduced per mole of nuclease. No evidence of unreacted tryptophan could be detected in fluorescence emission spectra, upon excitation at 295 nm. The NPS derivatives of *N*-acetyl-L-tryptophan likewise were nonfluorescent, upon excitation at 280 nm. There was no evidence from absorption spectra or from amino acid analysis to implicate reaction at any residue except the single tryptophan residue 140 of nuclease.

Enzymatic Activity of the Derivatives. In the spectrophoto-

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[1] Abbreviations used are: NPS, 2-nitrophenylsulfenyl; DNPS, 2,4-dinitrophenylsulfenyl; NCPS, 2-nitro-4-carboxyphenylsulfenyl; pdTp, deoxythymidine 3′,5′-diphosphate; BNPS-skatole, bromine adduct of 2-(2-nitrophenylsulfenyl)-3-methylindole.

TABLE I: Molar Absorptivities of NPS Derivatives of *N*-Ac-L-tryptophan.

Derivative	In 100% MeOH			In 80% Aqueous Acetic Acid					In Water, pH 7			
	λ_{max}[b]	ϵ	λ_{max}	ϵ	λ_{max}	λ_{max}	λ_{max}	ϵ	λ_{max}	ϵ	λ_{max}	ϵ
N-Ac-(NPS)-Trp	363	4000	283	14,100	362	3800	282	14,500	368	4050	283	14,800
N-Ac-(DNPS)-Trp	340[a]	8200	280	14,200	340[a]	8000	279	17,000	340[a]	8100	279	14,900
N-Ac-(NCPS)-Trp	358	4150	285	17,500	354	3700	285	17,300	365	4000	283	16,900

[a] Shoulder. [b] nm.

metric assay system of Cuatrecasas *et al.* (1967) NPS-, DNPS-, and NCPS-modified nuclease gave 48, 51, and 49% DNase activity and 49, 58, and 57% RNase activity, respectively. Maximal rates were obtained at the standard conditions of pH and of substrate concentration. The dependence of enzyme activity on calcium ion concentration showed a maximum at 10 mM both for the nuclease derivatives and for native nuclease (Figure 2).

Binding of the Specific Inhibitor Deoxythymidine 3',5'-Diphosphate (pdTp). Cuatrecasas *et al.* (1968) showed that native nuclease binds to an affinity column of Sepharose (agarose) 4B to which 3'-(4-aminophenylphosphoryl)deoxythymidine 5'-phosphate was covalently coupled; 2 mg of each of the three NPS derivatives of nuclease, dissolved in 1 ml of 0.05 M Tris-HCl (pH 8.8), containing 10 mM $CaCl_2$, was applied in separate experiments to a small column of pdTp-Sepharose (0.9 × 5 cm). Each derivative was adsorbed quantitatively and then eluted with full recovery, using aqueous NH_4OH (pH 11) to dissociate the enzyme–inhibitor complex.

The effect of binding of Ca^{2+} and pdTp in protecting against tryptic digestion (Taniuchi and Anfinsen, 1968) was studied for each of the derivatives. As shown in Table II, tryptic digestion promptly destroyed all enzymatic activity in the absence of ligands. The protective effect of bound ligands was as dramatic for the derivatives as for native nuclease. After 2 hr, however, the activity of NPS-nuclease fell to zero, while DNPS-, NCPS-, and native nuclease continued to have 10% activity.

Circular Dichroism. Intact nuclease has about 18% helical content accounting for characteristic minima in optical rotation at 233 nm and in ellipticity at 220 nm (Taniuchi and Anfinsen, 1968; Omenn *et al.*, 1970b). From the model based upon X-ray diffraction analysis, the residues 55–67, 99–107, and 122–133 are in α-helical conformation. Additional ordered structure occurs as a β-pleated sheet in the anti-

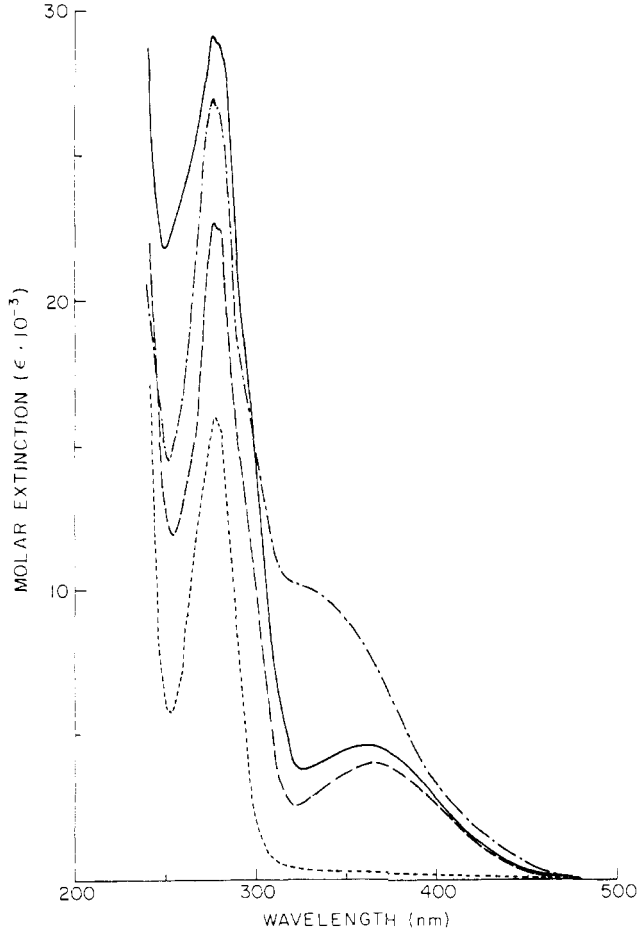

FIGURE 1: Ultraviolet absorption spectra of NPS- (— —), DNPS- (-·-·-·-), NCPS- (——) nucleases and native staphylococcal nuclease (----) in distilled water at pH 7.

TABLE II: Protection against Tryptic Digestion[a] by pdTp and Ca^{2+}.

	% Activity Remaining				
	30-min incubation		120-min incubation		5-hr incubation
	−pdTp	+pdTp	−pdTp	+pdTp	+pdTp
Native nuclease	0	35	0	10	10
NPS-nuclease	0	30	0	8	0
DNPS-nuclease	0	37	0	17	10
NCPS-nuclease	0	30	0	10	10
Performic acid oxidized nuclease[b]	0	8	0	0	0

[a] 10% w/w ratio of trypsin (Worthington) to nuclease or its derivative in absence or presence of 10^{-4} M deoxythymidine 3',5'-diphosphate and 10^{-2} M $CaCl_2$. All incubations were performed in 0.05 M Tris-HCl (pH 8.0) at 25°. [b] Omenn *et al.* (1970b).

TABLE III: Properties of Several Derivatives of Nuclease.

Derivative	Physical Data			Enzyme Activity		Immunological Reactivity	
	$E_{280}^{0.1\%}$	$[\theta]_{220}$	$[\eta]^c$	DNA	RNA	Immuno-diffusion	Turbidometric Assay
Nuclease	0.93	−9900	0.035	100%	100%	++	++
NPS derivatives							
NPS-nuclease	1.27	−5240	0.028	48	49	++	++
DNPS-nuclease	1.56	−5240	0.028	51	58	++	++
NCPS-nuclease	1.69	−5960	0.017	49	57	++	++
Oxidized derivatives							
BNPS-skatole[a]	0.70	−3100		18	12	++	++
Performic acid[b]	0.70	−3000		8	8	++	++
Hydrogen peroxide[b]	0.90	−3500		8	8	++	++

[a] Omenn *et al.* (1970a). [b] Omenn *et al.* (1970b). [c] Intrinsic viscosities in deciliters per gram.

parallel form, involving three extended chains within residues 12–36. As shown in Figure 3, the circular dichroic spectra of these NPS derivatives lack the characteristic trough at 220 nm, but the degree of negative ellipticity is greater than that remaining in the nuclease derivatives whose tryptophan had been oxidized to an oxindole (Omenn *et al.*, 1970a,b). Among the three NPS derivatives, there is no difference between NPS- and DNPS-nuclease, but the circular dichroism of the NCPS-nuclease is somewhat greater.

Studies of Viscosity. Values of intrinsic viscosity were obtained from the intercepts of plots of reduced viscosity against protein concentration. All three nuclease derivatives have intrinsic viscosities similar to that of native nuclease (Heins *et al.*, 1967) (Table III). Although the reduced viscosities of the NPS-nucleases are more strongly dependent on concentration, indicating enhanced intermolecular interaction, this effect was not significant in the concentration range used for all other physical, enzymatic, and immunochemical studies of these derivatives.

Immunochemical Properties. Each NPS derivative of nuclease gave a precipitin line of identity with native nuclease in agar double immunodiffusion against anti-nuclease antibody (Figure 4). Quantitative turbidometric assay of the precipitation reaction showed no significant differences among the three derivatives and nuclease.

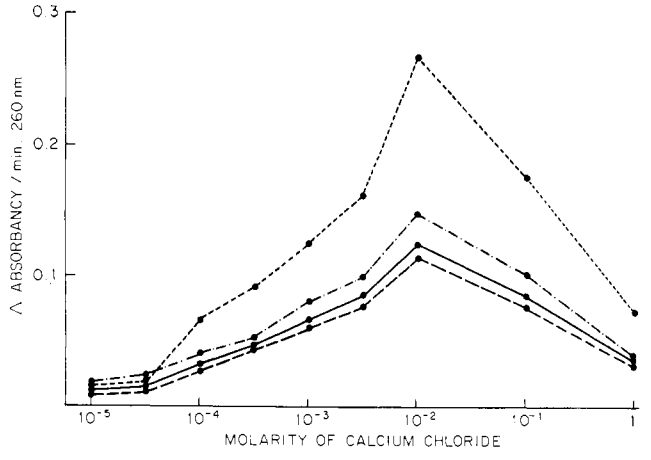

FIGURE 2: Spectrophotometric assay of enzymatic activity of NPS- (— — —), DNPS- (·-·-·), NCPS- (——), and native nuclease (----) as a function of calcium ion concentration. All assays were performed at room temperature with DNA (heat denatured, 100 μg/ml) as substrate, in Tris-HCl buffer, 0.05 M, pH 8.8.

Discussion

Oxidizing agents which convert methionine into methionine sulfone and tryptophan to kynurenine have drastically altered

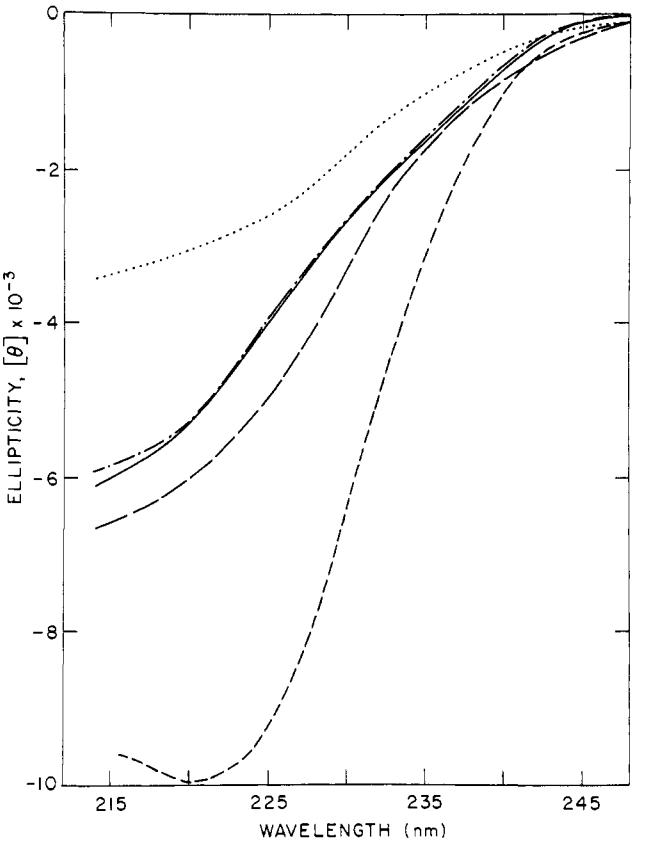

FIGURE 3: Circular dichroic spectra of NPS- (——), DNPS- (·-·-·), and NCPS- (— — —) nuclease at concentration of 3–4 μM at pH 8.0, 27°, with 10-mm cell path. Spectra of native nuclease (----) and of nuclease-Trp^{140}-oxindole (· · · ·) are given for comparison.

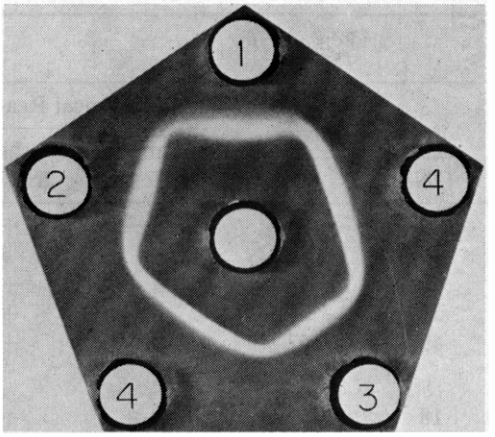

FIGURE 4: Ouchterlony double immunodiffusion in agar, with anti-nuclease antiserum in the center well and with NPS-nuclease (1), nuclease (4), DNPS-nuclease (2), and NCPS-nuclease (3) in the outer wells at concentrations of about 200 μg/ml.

the energies of conformational stability of staphylococcal nuclease and have destroyed most of the enzymatic activity (Omenn *et al.*, 1970a,b). In such derivatives, the normally hydrophobic methionine and tryptophan residues have been made hydrophilic and might serve to distort inside–outside relationships in the conformation of the protein. NPS reagents, which add bulk to the single tryptophan residue without altering its hydrophobic nature, provided a more subtle test of the effects of local distortion of nuclease conformation.

Reaction with tryptophan required reversible acid denaturation of the enzyme, to make the tryptophan accessible. Under these conditions, a five- to tenfold molar excess of each reagent gave complete reaction. No major differences were encountered in comparing numerous properties of the series of NPS-tryptophanyl-nucleases, but the NCPS derivative had greater negative ellipticity and both NCPS- and DNPS-nucleases had longer lasting protection from bound ligands than did NPS-nuclease in the tryptic digestion experiments. All retained 100% immunological cross-reactivity and about 50% DNase and RNase activity. Binding of deoxythymidine 3′,5′-diphosphate and Ca^{2+} stabilized the conformations of these derivatives as completely as that of the native enzyme against tryptic digestion. By contrast, modified nucleases in which the tryptophan was converted to the oxindole were able to bind pdTp and Ca^{2+}, but the protective effect of the ligands was incomplete, allowing loss of all activity within 60 min (Omenn *et al.*, 1970b). The specific activity of the oxidized derivatives, of course, was much lower than that of these NPS derivatives (Table III). We consider susceptibility to proteolytic digestion as a dynamic test of conformational stability. Another point of interest is the equal diminution of activity against DNA and RNA as substrates in the current study. Earlier modifications of nuclease by acetylation, nitration, oxidation, substitution of Sr^{2+} for Ca^{2+}, and the use of denaturants caused greater loss of RNase activity than of DNase activity (see Omenn *et al.*, 1970a). Comparative kinetic studies of the enzymatic action of nuclease and of its derivatives may elucidate fine differences in binding of DNA and of RNA and in the respective hydrolytic mechanisms.

The marked change in circular dichroism (Figure 3) confirmed our expectation from the X-ray diffraction-based model (Arnone *et al.*, 1969, 1971) that considerable distortion of the α helices of the carboxy-terminal part of nuclease would be necessary to accommodate the bulky NPS groups on tryptophan upon refolding of the chemically modified enzyme. The circular dichroic evidence for residual ordered conformation without the minimum at 220 nm characteristic of α-helical structure suggests the possibility that the extensive pleated sheet or β barrel formed at the opposite side of the molecule (residues 12–26) may persist despite these modifications of the tryptophan residue. The exact orientation of the indole ring, situated perpendicular to the surface of the molecule between two stretches of α-helical conformation at the carboxy terminus, has not been fixed with certainty in the molecular model of nuclease, because of reduced intensity in the electron density map at the chain ends. However, with the knowledge that reversible denaturation of the enzyme is required to permit reagents to attack the C-2 atom of the tryptophan indole ring, it is likely that the five-membered ring, not the six-membered ring, is situated more to the interior of the three-dimensional structure.

In considering the role of the carboxy-terminal portion of nuclease, we should note that a fragment of nuclease containing residues 1–126 has no enzyme activity and no ordered conformation (Taniuchi and Anfinsen, 1969). However, overlapping fragments containing the carboxy-terminal sequence (6–48 plus 49–149 or 1–126 plus 49–149 or 1–126 plus 99–149) confer ordered structure and about 8–10% of the specific enzymatic activity of nuclease, by forming a noncovalently bound complex (Taniuchi and Anfinsen, 1968, 1969; Taniuchi, 1970). The function of tryptophan within this sequence has been explored with fragments of nuclease synthesized by the solid-phase method. A fragment (residues 99–148) containing phenylalanine in place of tryptophan at residue 140 forms such an enzymatically active complex with the nuclease fragment 1–126 (Parikh and Anfinsen, 1970), supporting the conclusion that tryptophan is not essential for the catalytic function of nuclease, but that tryptophan plays a sensitive structural role in the conformation of the enzyme.

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Kinetic Evidence for Propagation of Conformational Changes in the α Subunit to the β Subunit of Hemoglobin*

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ABSTRACT: The reaction of *p*-mercuribenzoate with the β93 SH groups of the artificial half-methemoglobins has been studied by the stopped-flow method, to investigate propagation of conformational change in a subunit to the neighboring subunits. The second-order rate constants for the reaction have been evaluated from kinetic plots. It is found, from comparison of the rate data, that the reactivity of the β subunit is primarily dependent on ligation of the β subunit and is also dependent on ligation of the neighboring α subunits.

With an assumption that some changes in the rate constant reflect alterations in conformation of the β subunit, the present results suggest that the β subunit takes four different conformations, named O, P, Q, and R, depending on the state of the α subunit. Deoxy β subunit takes R conformation with deoxy α and Q with met α. The conformation of met β subunit is O with oxy and met α and changes into P when the α subunit becomes deoxygenated. Thus, ligation of the α subunit affects the conformation of the β subunit. Some implications are presented on the allosteric mechanism.

Two different models have been presented for the molecular mechanism of the cooperative binding of substrate to allosteric proteins. In the Monod–Wyman–Changeux model, the subunits of a protein molecule take two different conformations, arranged in a symmetrical fashion, and the protein molecule is assumed to maintain symmetry during the conformational changes (Monod *et al.*, 1965). The Koshland–Némethy–Filmer model, on the other hand, assumes progressive or sequential changes in the subunit conformation, only the subunit binding a ligand being able to transform its conformation (Koshland *et al.*, 1966).

Both models can equally well explain the experimental saturation curves of hemoglobin. In order to obtain further insights in the allosteric interactions, it is necessary to investigate the subunit conformation during the allosteric transitions and several studies have already appeared using some physical techniques as well as some chemical methods (Ogawa and McConnell, 1967; Hayashi *et al.*, 1967; Ogawa *et al.*, 1968; Shulman *et al.*, 1969; Antonini and Brunori, 1969; Brunori *et al.*, 1970). All the results indicated that the conformation of a subunit changed when the subunit was liganded. On the possibility of propagation of the conformational changes to the neighboring subunits, however, there have been published two different conclusions. Most authors did not observe such propagation while some reported existence of the propagation. To study this problem, we measured, in the present paper, the rate constant of the reaction of PMB[1] with the β93 SH groups of the artificial half-methemoglobins, since the rates of reactions of various sulfhydryl reagents with the β93 SH groups of oxyhemoglobin have been known to be greatly different from those for deoxyhemoglobin (Antonini and Brunori, 1969, and the literatures listed therein). Our results have suggested propagation of conformational changes in the α subunit to the β subunit.

Experimental Section

Isolation of α and β Chains. Human adult hemoglobin was prepared from fresh blood by lysing washed red cells with 1–1.5 volumes of water. The mercurated α chains, α_{PMB}, were prepared by the method of Geraci *et al.* (1969) and the mercurated β chains, β_{PMB}, were obtained according to the procedure described by Bucci and Fronticelli (1965). The α chains were rendered free of mercury by washing the α_{PMB} chains adsorbed on CM-cellulose column, equilibrated with 0.01 M phosphate buffer (pH 6.7), with 0.015 M mercaptoethanol. Removal of mercury from the β_{PMB} chains was carried out by the method of Tyuma *et al.* (1966).

Preparation of Hybrid Half-Methemoglobins. The α chains

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[1] Abbreviation used is: PMB, *p*-mercuribenzoate. The superscripts +, O_2, and +CN, written to the right of α_2 and β_2, represent that the indicated subunits are in the met, in the oxygenated, and in the cyanmet form, respectively.