# Homogeneity and Variability in the Structure of Azurin Molecules Studied by Fluorescence Decay and Circular Polarization†

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ABSTRACT: The fluorescence decay of apoazurin derived from *Pseudomonas aeruginosa* is monoexponential. By this criterion the population of molecules of apoazurin is homogeneous. The emission anisotropy factor and the absorption anisotropy factor at the red edge of the absorption band assume similar values, showing that the tryptophan residue in apoazurin has the same asymmetric environment both in the ground and excited states. This finding suggests tight packing of the protein at the tryptophan environment. Native azurin does not decay monoexponentially. Moreover, comparison between the quantum yield calculated from the decay kinetics and the one measured directly shows that the majority of the azurin molecules are not fluorescent. There is thus variability in the structure of azurin molecules with an equilibration time that is longer than the fluorescence lifetime. Different asymmetric environment was found for the tryptophan residue in oxidized and reduced holoprotein and in apoazurin, as studied by the circular polarization of the fluorescence. $D_2O$ increases the fluorescence lifetime of apoazurin by 6%, compared to the lifetime in $H_2O$ solution; therefore water molecules may have access to the tryptophan residue, though the latter is situated in a hydrophobic environment.

Azurins are bacterial copper containing proteins which function as electron mediators in the energy conversion system. These are relatively small proteins, of about 14,000 molecular weight, and contain their single copper ion in a unique coordination environment as the redox active center (Sutherland and Wilkinson, 1963; Ambler, 1963; Ambler and Brown, 1967).

Azurins from several bacterial strains contain a single tryptophan residue, the position of which is fully conserved (Ambler, 1971); therefore this residue probably has a functional role in these proteins. The fluorescence properties of the tryptophan residue are unique; the emission peak is at an unusually short wavelength, 308 nm, indicating that the environment of the tryptophan residue is highly hydrophobic (Finazzi-Agrò et al., 1970). At neutral pH, the same emission spectrum is obtained for azurin and a variety of azurin derivatives, such as apoazurin (from which $Cu^{2+}$ has been removed), and complexes of apoazurin with $Cu^{+}$, $Ag^{+}$ and $Hg^{2+}$; the quantum yields vary, however, widely (Finazzi-Agrò et al., 1973). The emission spectrum changes significantly upon acidification or denaturation. Hence, the tryptophan fluorescence has been found to be a useful tool in the study of structural problems of azurin.

Recently, Lakowicz and Weber (1973) have shown by the method of fluorescence quenching by oxygen that azurin molecules undergo structural fluctuations which are fast relative to the fluorescence decay of the protein. Other proteins were shown to behave similarly. This conclusion was based on the findings that the quenching of the tryptophan fluorescence is dynamic and not static, indicating that the oxygen molecules are capable of diffusing toward the buried tryptophan residue on the time scale of the fluorescence lifetime. The above method is, however, not suitable for detecting more permanent structural fluctuations, i.e., variability in the structure of the protein molecules which persists for periods longer than the fluorescence lifetime. Such variability can be detected by other fluorometric techniques. In the following we apply the techniques of fluorescence decay and circular polarization of luminescence, CPL,[1] for this purpose.

The fluorescence decay of a homogeneous population of excited chromophores is expected to be monoexponential. If the population of the molecules of the protein has some variability in conformation which persists for a period longer than the lifetime of the excited tryptophan, and if the quantum yield of the tryptophan residue is affected by this variability in molecular conformation, the fluorescence decay cannot be expected to be monoexponential any longer. For quenching processes that compete kinetically with the radiative decay of the excited state, the observed decay lifetime of a specific chromophore, $\tau$, is related to the radiative lifetime, $\tau_0$, and the quantum yield, $q$, by the relation $\tau = q\tau_0$. Thus, deviation from monoexponential decay in a protein with a single tryptophan discloses heterogeneity in the molecular conformation. If the fluorescence is fully quenched in some of the protein molecules, these will not show up in the decay curve; they may, however, be disclosed by comparing the experimentally found quantum yield to that expected from the fluorescence decay measurements, as will be shown below.

Under suitable conditions the heterogeneity of luminescent molecules can also be demonstrated by the spectral behavior of the circular polarization of the luminescence, CPL. The CPL of a chromophore reflects its asymmetry in the electronically excited state from which emission occurs and is therefore the excited state analog of CD, which re-

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[1] Abbreviations used are: NATA, *N*-acetyl-L-tryptophanamide; CPL, circular polarization of luminescence.

flects the asymmetry in the ground state (Gafni and Steinberg, 1972; Schlessinger and Steinberg, 1972; Steinberg, 1975; Steinberg et al., 1974). The CPL is conveniently expressed by the emission anisotropy factor, $g_{em}$, defined as $g_{em} = \Delta f/(f/2)$, where $\Delta f$ is the intensity of the circularly polarized fraction of the emitted light (defined as positive for left-handed circular polarization) and $f$ is the total intensity of the emitted light. Provided the transitions involved are not forbidden, the anisotropy factor is expected to be constant across each electronic band. Since in condensed media the emission of a pure homogeneous substance involves generally a single electronic transition, $g_{em}$ is expected to be constant across the emission band under such circumstances (Moscowitz, 1965; Steinberg et al., 1974). If the substance studied is heterogeneous with respect to the emission spectra and to the asymmetry of the constituent molecules, deviations from constancy of $g_{em}$ will be observed (Steinberg, 1975; Steinberg et al., 1974; Schlessinger et al., 1974a).

In the present study, the above criteria have been applied to azurin and some of its derivatives. While apoazurin behaves as a homogeneous protein, heterogeneity is demonstrated in the population of molecules of holoazurin under a variety of conditions. The finding that a protein with a single tryptophan may show multiexponential kinetics for its fluorescence decay has implication on the interpretation of the fluorescence studies of proteins in general.

## Experimental Section

*Materials.* Azurin was prepared from *Pseudomonas aeruginosa* and purified by the procedure described by Ambler (1963, 1967). The ratio of the optical density at 625 and at 280 nm was 0.48 in the batch studied. Apoazurin was prepared by dialysis of the protein against 0.02 *N* KCN solution buffered at pH 7.5 by 0.02 *M* phosphate. Reduction of the $Cu^{2+}$ in azurin was performed by a small excess of ascorbate ions, which were subsequently removed by gel filtration on G-25 Sephadex, or by Pt-black catalyzed reduction with $H_2$.

Ultrapure guanidinium hydrochloride was a product of Schwarz/Mann. Purified *N*-acetyl-L-tryptophanamide (NATA) was a kind gift from Dr. M. Shinitzky. Deuterium oxide ($D_2O$) was purchased from Roth (Karlsruhe, Germany). All other chemicals were of Analar or Puriss grade. Measurements were performed on solutions prepared in three times distilled water.

### *Methods*

*Absorption and Fluorescence.* Absorption measurements were made on a Zeiss Model PMQ II and Cary 15 spectrophotometers. Corrected fluorescence spectra were obtained with a Perkin-Elmer, Model MPF-3L, spectrofluorometer. The optical density of the solutions was about 0.1 at the wavelength of excitation. Quantum yields were measured relative to NATA. Excitation was done at the same wavelength for the standard NATA, and the protein. Relative quantum yields were determined by the ratio of the area under the corrected fluorescence emission bands divided by the corresponding ratio of the absorbance. A bandwidth of 1.5 nm was used for both the excitation and emission monochromators.

The parameters obtained for the fluorescence decay function are related to the quantum yield of the fluorescence (Hazan, 1973). Suppose the decay of the fluorescence intensity, $i(t)$, is represented by a sum of exponentials:

$$i(t) = \sum_{k=1}^{p} \alpha_k \exp(-t/\tau_k) \tag{1}$$

The quantum yield of the system, $q$, is given by the amount of light emitted divided by the total number of excited molecules. Let $N_k$ be the number of excited molecules with a lifetime $\tau_k$, radiative lifetime $\tau_k^0$, and quantum yield $q_k$ and let there be $N_{nf}$ molecules which absorb but are nonfluorescent. The total number, $N$, of molecules which absorb light is thus $N_{nf} + \Sigma_{k=1}^{p} N_k$. The quantum yield is given by

$$q = \sum_{k=1}^{p} N_k q_k / N \tag{2}$$

Remembering that $q_k = \tau_k/\tau_k^0$ (Hazan, 1973)

$$q = (\sum_{i=1}^{p} N_k \tau_k/\tau_k^0)/N \tag{3}$$

Upon rearranging, and remembering that $N_k = \alpha_k \tau_k^0$ (Grinvald and Steinberg, 1974a)

$$\frac{\sum_{k=1}^{p} N_k}{N} = \frac{q \sum_{k=1}^{p} N_k}{\sum_{k=1}^{p} N_k \tau_k/\tau_k^0} = \frac{q \sum_{k=1}^{p} \alpha_k \tau_k^0}{\sum_{k=1}^{p} \alpha_k \tau_k} \tag{4}$$

In the cases studied (Badley and Teale, 1969; Weinryb and Steiner, 1968) it was found that the radiative lifetime of tryptophan is constant under a variety of circumstances. Let us call this constant $\tau^0$. Thus

$$\frac{\sum_{k=1}^{p} N_k}{N} = \frac{q\tau^0 \sum_{k=1}^{p} \alpha_k}{\sum_{k=1}^{p} \alpha_k \tau_k} \tag{5}$$

The fraction of nonfluorescent molecules, $F_{nf}$, is given by

$$F_{nf} = \frac{N_{nf}}{N} = 1 - \frac{\sum_{k=1}^{p} N_k}{N} = \frac{\sum_{k=1}^{p} \alpha_k \tau_k - q\tau^0 \sum_{k=1}^{p} \alpha_k}{\sum_{k=1}^{p} \alpha_k \tau_k} \tag{6}$$

The relative quantum yield of the protein, $q_r$, is measured with a standard which has a quantum yield $q_s$, thus $q = q_r q_s$. Whenever the standard has a single exponential decay with lifetime $\tau_s$ and molecules which absorb also fluoresce, $q_s = \tau_s/\tau^0$. The original $\alpha_k$ values can be normalized, i.e., $\Sigma_{k=1}^{p} \alpha_k = 1$. Thus we obtain

$$F_{nf} = \frac{\sum_{k=1}^{p} \alpha_k \tau_k - q_r \tau_s}{\sum_{k=1}^{p} \alpha_k \tau_k} \tag{7}$$

Equation 7 is very useful for lifetime measurements of proteins. It has to be used with care after verifying that the assumption which was mentioned above is valid, i.e., that $\tau_0$ is the same for all species involved.

*Measurement of Fluorescence Decay.* The instrument for the measurement of fluorescence decay kinetics was of the type described by Hundley et al. (1967). A useful modification to overcome drift problems has been introduced in our laboratory; in our measurements the profiles of the excitation lamp and the emitted fluorescence are collected alternatingly at frequent time intervals (Hazan et al., 1974).

The fluorescence decay data were analyzed by the method of nonlinear least squares (Grinvald and Steinberg,

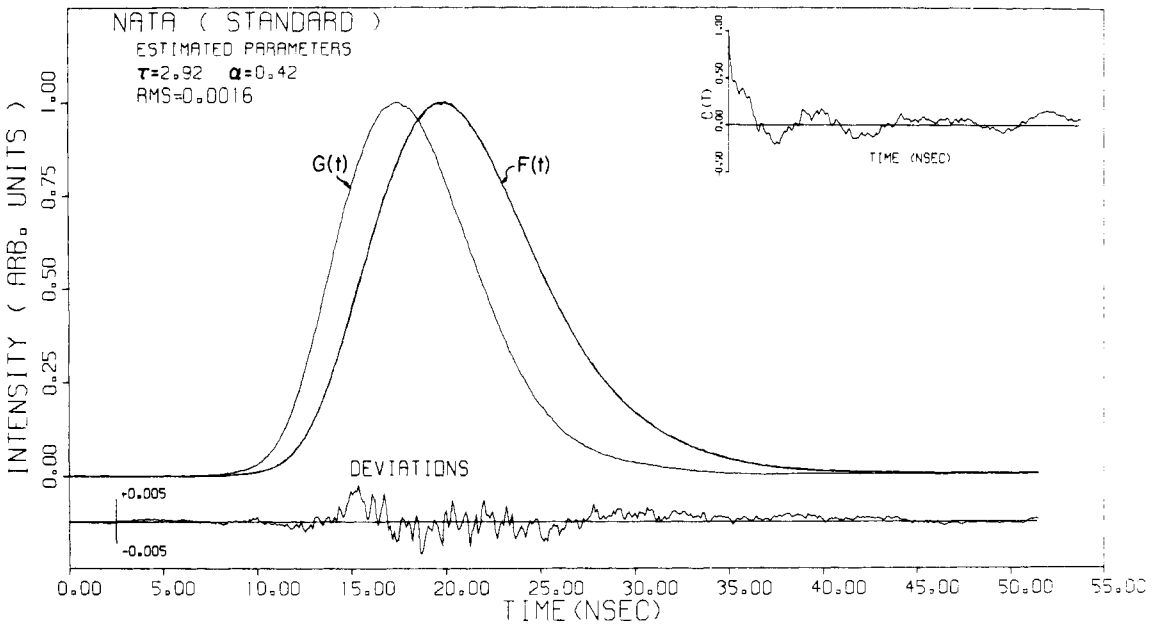


FIGURE 1: Test of the fluorescence decay instrument by *N*-acetyl-L-tryptophanamide (NATA) used as a standard. Concentration, $5 \times 10^{-5}$ *M* in 0.1 *M* phosphate buffer (pH 7.0). Excitation wavelength, 296 nm. Emission filter, Corning 7-60. $G(t)$, excitation light profile; $F(t)$, two superimposed curves; the noisy curve is the experimental fluorescence decay curve, the smooth curve is the calculated fluorescence decay assuming a monoexponential decay function for the standard substance. The noisy curve at the bottom of the figure represents the deviations between the experimental and the calculated curves. Insert: the autocorrelation function of the deviations. The fit to a single exponent is excellent as seen by the very low RMS ($16 \times 10^{-4}$) and the shape of the autocorrelation function.

1974a,b). The decay kinetics were assumed to be either monoexponential or biexponential functions, the amplitudes and the lifetimes being taken as free parameters to be estimated by fitting the assumed decay function to the experimental data. We have tested the instrumental setup by using highly purified *N*-acetyl-L-tryptophanamide as a fluorescence decay standard. The result is presented in Figure 1. The data could be fitted to a monoexponential decay function with a very low root mean square, RMS,[2] deviation of $1.6 \times 10^{-3}$. The autocorrelation function (Grinvald and Steinberg; 1974a,b) (see Figure 1) shows that the data are almost free of systematic error. Each set of experiments with protein solutions was accompanied with a measurement of the standard. In all of the experiments two exponents were sufficient to fit the experimental data. This does not mean that the true decay function is not more complex; it is only that the quality of the data does not permit accurate and reliable analysis for more than two components in such a case (Grinvald and Steinberg, 1974a). Such behavior is, however, a clear indication of heterogeneity in the system studied. The range of stochastic error in the values of the parameters of any decay function can be estimated (Grinvald and Steinberg, 1974a). Other sources of errors are due to minor systematic deviation and to timing calibration (less than 2% in our instrument). The range of error quoted in Table I is an estimate of all these possible errors.

*CPL Measurements.* The instrument for the measurement of CPL was built in our laboratory and has been described elsewhere (Steinberg and Gafni, 1972; Steinberg et al., 1974; Schlessinger et al., 1975). The estimated uncertainty in the value of $g_{em}$ is $\pm 5 \times 10^{-5}$.

All experiments were conducted at room temperature (22 ± 0.5°).

## Results and Discussion

### *Apoazurin*

*Neutral pH.* The spectra of fluorescence emission and fluorescence excitation of apoazurin are presented in Figure 2. The absorption and emission spectra are similar to those published previously (Finazzi-Agrò et al., 1970), except that the present data have been obtained at a higher spectral resolution. This is probably the reason why the peak at 298 nm could not be resolved previously. The quantum yield of apoazurin was measured relative to NATA. A value of 1.5 was obtained. The lifetime of NATA (2.9 nsec) is about the same as that of tryptophan (2.6 nsec) at neutral pH, thus the quantum yield of NATA and tryptophan should be of similar magnitude (Weinryb and Steiner, 1968), i.e., 0.13 (Chen, 1967) or 0.20 (Teale and Weber, 1957). The absolute quantum yield of apoazurin thus falls in the range 0.20–0.30. This value is significantly higher than the value of 0.1 reported previously (Finazzi-Agrò et al., 1970). The reason for the discrepancy is not clear.[3] The unusual emission of the tryptophan residue at 308 nm and the well-resolved shoulder at 298 nm most probably reflect a highly hydrophobic environment for the tryptophan (Konev, 1967). The excitation spectra for light emitted at 300, 310, 340, and 360 nm are identical; thus if dual simultaneous emission from both the $^1L_a$ and $^1L_b$ levels exists (Song and

[2] RMS = $(1/n)(\Sigma_{i=1}^{n}[F_c(t_i) - F(t_i)]^2)^{1/2}$, where $F(t_i)$ is the measured fluorescence intensity at the $i$th time interval, $t_i$, and $F_c(t_i)$ is the fluorescence intensity at $t_i$ calculated from the assumed decay function.

[3] Our measurements were made with azurin extracted from *Pseudomonas aeruginosa* but that of Finazzi-Agro et al. was from *Pseudomonas fluorescence*. These two species were confused in the past (Ambler, 1973) and it is therefore uncertain which species the above authors used.

Table I: Fluorescence Decay Data for *N*-Acetyl-L-tryptophanamide and Azurin under Various Conditions.[a]

| Sample[b] | One Component | | | | Two Components | | | |
|---|---|---|---|---|---|---|---|---|
| | $\lambda_{ex}$ (nm) | Emission Range[c] | $\langle\tau\rangle$[d] | RMS[e] ($\times 10^{-4}$) | $\tau_1$[f] | $\tau_2$[f] | $\alpha_1$[g,h] | RMS[i] ($\times 10^{-4}$) |
| NATA | 280 | 310–390 | 2.9 | 16 | | Single exponential | | |
| | 296 | 310–390 | 2.9 | 16 | | Single exponential | | |
| | 296 | >370 | 2.9 | 17 | | Single exponential | | |
| NATA in $D_2O$ | 280 | 310–390 | 3.2 | 17 | | Single exponential | | |
| NATA in 6 *M* Gdn·HCl[j] | 280 | 310–390 | 2.9 | 18 | | Single exponential | | |
| NATA (pH 1.5) | 280 | 310–390 | 0.9 | 18 | | Single exponential | | |
| NATA + 0.1 *M* quencher[k] | 280 | >310 | 0.54 | 15 | | Single exponential | | |
| Apoazurin | 280 | 320–380 | 4.8 | 13 | | Single exponential | | |
| | 295[l] | 320–380 | 4.7 | 25 | | Single exponential | | |
| | 280 | >370 | 4.7 | 30[m] | | Single exponential | | |
| Apoazurin in $D_2O$ | 280 | >310 | 5.1 | 20 | | Single exponential | | |
| Holoazurin | 280 | >310 | 4.0 | 208 | 4.7 | 0.3 | 0.35 | 14 |
| | 295[l] | >310 | 3.5 | 219 | 4.5 | 0.8 | 0.44 | 21 |
| Reduced azurin | 280 | >310 | 4.4 | 140 | 4.8 | 0.2 | 0.43 | 15 |
| | 295[l] | >310 | 4.0 | 227 | 4.8 | 0.4 | 0.41 | 20 |
| Apoazurin pH 1.5 | 295 | 320–380 | 2.1 | 152 | 4.1 | 1.1 | 0.17 | 15 |
| Apoazurin +6 *M* Gdn·HCl[j] | 296 | 320–380 | 2.5 | 90 | 3.4 | 1.2 | 0.38 | 15 |
| Holoazurin +6 *M* Gdn·HCl[j] | 296 | 320–380 | 2.3 | 71 | 3.2 | 1.3 | 0.36 | 15 |
| | 296 | >370 | 3.0 | 94 | 4.2 | 1.7 | 0.36 | 13 |
| Apoazurin +0.1 *M* quencher[k] | 280 | >310 | 4.1 | 31 | 4.7 | 3.0 | 0.60 | 16 |

[a] Protein concentration, $2.5 \times 10^{-5}$ *M*; NATA concentration, $5 \times 10^{-5}$ *M*. For the range of error of these parameters see also the paragraph on data analysis in the Experimental Section. [b] Unless otherwise stated, measurements were carried out in 0.1 *M* phosphate buffer (pH ~7). [c] Four filters were used; Schott KV-370 (cut off at 370 nm), a piece of commercial transparent nonfluorescent glass (cut off at 310 nm), Corning 7-60 (transmission range, 310–390 nm), and Kodak 18A (transmission range, 320–380 nm). [d] Estimated error is about ±0.1 nsec. If the decay is not monoexponential our method of analysis calculates an average lifetime $\langle\tau\rangle$. The area under the calculated one-component decay curve is almost equal to the area under the experimental decay curve, i.e., $\alpha\langle\tau\rangle = \alpha_1\tau_1 + \alpha_1\tau_2$. ($\alpha$ can be calculated from the table.) [e] The RMS obtained upon fitting the decay of NATA to a single component is $10-18 \times 10^{-4}$, depending on how long the experiment was carried out. Whenever the RMS is appreciably greater it is evident that the decay is definitely not monoexponential. [f] Estimated error is about ±0.15 nsec. [g] The values of the $\alpha$'s were normalized, i.e., $\alpha_1 + \alpha_2 = 1$. [h] Estimated error is 5–15% depending upon the values of the rest of the parameters. Because of the correlation which exists among the parameters positive error in $\tau_1$ is associated with positive error in $\tau_2$ and usually negative error in both $\alpha_1$ and $\alpha_2$. The error in the ratio $\alpha_1/\alpha_2$ may be as high as 30%, however, the estimated error in the ratio $\alpha_1\tau_1/\alpha_2\tau_2$ which correspond to the ratio of light emitted by two components is less than ±10%. [i] In all cases the RMS is below $20 \times 10^{-4}$, so the fit to a biexponential decay is satisfactory. The true decay functions may be more complex, but the quality of the data does not justify further refinement. [j] Gdn·HCl denotes guanidine hydrochloride. [k] The quencher is *N*-methylpyridinium perchlorate. [l] Some scattered light may influence the parameters of the decay function; we therefore feel that it is not certain whether the results are significantly different from those obtained upon excitation at 280 nm. [m] Significant contribution of fluorescence from the filter was probably the reason for the relatively high RMS value. (It may be seen in Figure 2 that very little fluorescence emitted by the protein exceeds the wavelength of 380 nm.)

Kurtin, 1969; Andrews and Forster, 1974) the population of the excited level must be thermally equilibrated.

The absorption and emission anisotropy factors, $g_{ab}$ and $g_{em}$, respectively, of apoazurin are also included in Figure 2. The values of $g_{ab}$ were calculated from the data of Tang and Coleman (1968) by the expression, $g_{ab} = \Delta\epsilon/\epsilon$, where $\Delta\epsilon$ is the difference in the extinction coefficients for right-handed and left-handed circularly polarized light, and $\epsilon$ is the average extinction coefficient. The value of $g_{ab}$ is highly variable across the absorption spectrum, which reflects the multitude of chromophores and electronic transitions involved. In contrast, $g_{em}$ at neutral pH is constant across the emission band, which is a necessary, though not sufficient, requirement for a homogeneous collection of chromophores with an allowed transition (Moscowitz, 1965; Steinberg, 1975; Steinberg et al., 1974).

Another interesting fact regarding the anisotropy factors is the similarity between the value of $g_{em}$ and the value of $g_{ab}$ at the red edge of the absorption band, the spectral region where the transition to the first singlet excited state of the tryptophan residue predominates. This finding indicates that the conformation of the indole residue and its interaction with its local environment are very similar in the ground state and in the excited state. Similar behavior was found in the enzyme staphylococcal nuclease, which also contains a single tryptophan residue (Steinberg et al., 1974). An important consequence of this finding is that the data which are obtained from fluorescence measurements in the cases discussed are completely relevant to the ground state. This was not found to be so with some fluorescent dyes which are bound to proteins (Schlessinger and Steinberg, 1972; Steinberg, 1975), or with fluorescent haptens which are bound to the corresponding antibodies (Schlessinger et al., 1974a). In these cases there are marked differences between $g_{em}$ and $g_{ab}$ at the long wavelength absorption band, reflecting changes in the asymmetry of the chromophores which occur upon electronic excitation. It seems that the protein tertiary structure around the indole residue impedes conformational changes which may occur due to the electronic excitation. Such changes were observed in fluid solvents in diketopiperazines which contain tryptophan, but do not take place in very viscous solvents, which may mimic the "frozen" conformation of indole side chains in the protein molecules discussed (Schlessinger et al., 1974b; Steinberg et al., 1974b).

The fluorescence decay data for apoazurin are presented in Figure 3, and the results are summarized in Table I. The fluorescence was collected after passing one of three different filters, one cutting off at 310 nm (a piece of nonfluorescent window glass), the second at 370 nm (Schott KV-370), and the third with a transmission window in the spectral range 310–390 nm (Corning 7-60). In all cases the

decay of apoazurin was found to be monoexponential with a lifetime of 4.7 nsec. A comparison between the trace of the residuals and the autocorrelation function of apoazurin and our fluorescence decay standard, NATA (see Figure 1), shows that the decay of the fluorescence of this protein is as close to monoexponential as can be detected within experimental error. It should be noted that this is not a trivial result, since other proteins which contain a single tryptophan residue, like human serum albumin (De-Lauder and Wahl, 1971) and naja toxin (A. Grinvald, unpublished results) do not fulfill this requirement. To the extent that the tryptophan fluorescence is a reflection of the environment of this residue, the above finding indicates that all apoazurin molecules in solution have identical conformations in the regions affecting the tryptophan. The above result does not contradict the possibility of fast fluctuation in conformation which occur many times during the lifetime of fluorescence emission (Lakowicz and Weber, 1973), nor do they exclude the possibility of the occurrence of different conformations which by chance have identical decay times.[4]

The decay function of apoazurin is monoexponential upon excitation at 280 nm where tyrosines absorb significantly, with a decay time that is very similar to that obtained upon excitation at 295 nm. This finding shows that energy transfer from the tyrosine residues to the tryptophan residue in the molecule is either very fast or totally absent, since slow transfer would result in deviation from monoexponential decay. The relaxation processes responsible for the Stokes shift of azurin fluorescence are very fast, the corresponding rate constant estimated to exceed $10^{11}$ $sec^{-1}$. This is deduced from the fact that no buildup was observed in the fluorescence at the red edge of the emission. Slow relaxation processes which cause a subnanosecond delay of the red edge of the emission of a protein have been described recently (Grinvald and Steinberg, 1974b). If the emission of apoazurin is from both the excited states $^1L_a$ and $^1L_b$ (Andrews and Forster, 1974), the monoexponential decay proves that both these energy levels are thermally equilibrated because it is unlikely that they will have similar lifetimes.

*Acid pH.* The emission spectrum of apoazurin changes markedly on lowering the pH to 1.5 by HCl (Finazzi-Agrò et al., 1970, and Figure 4), reflecting a pronounced change in molecular conformation. Upon excitation at 280 nm the emission shifts to longer wavelengths, the peak being at 343 nm, with a pronounced shoulder at about 310 nm. The shoulder is most probably due to tyrosine emission, since it disappears on excitation at 295 nm, where tyrosine absorption is negligible. Evidently, the efficient transfer of excitation energy from the tyrosines to the tryptophan residue at neutral pH decreases or stops in acid pH, probably as a result of the change in molecular conformation.

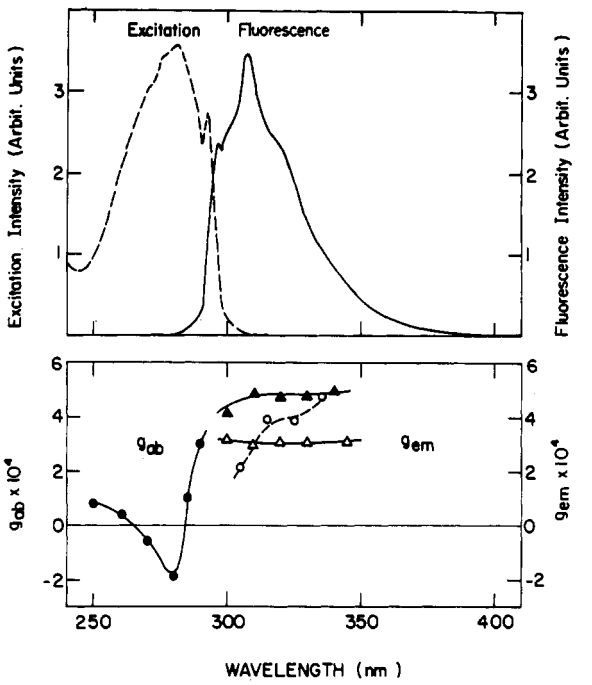


FIGURE 2: Spectroscopic data for azurin. (—) Fluorescence spectra of azurin and apoazurin (excitation wavelengths, 270 or 295 nm; spectral bandwidth, 1.5 nm for both excitation and emission monochromators). Excitation spectra of azurin and apoazurin (emission wavelengths, 300, 310, 340, and 360 nm; resolution, 1.5 nm for the excitation and emission monochromators). Protein concentration, $10^{-5}$ *M* in 0.05 *M* phosphate buffer (pH 7.5). Emission anisotropy factor, $g_{em}$, of azurin (▲); apoazurin (Δ); and reduced azurin (○). (Protein concentration, 2.1 × $10^{-4}$ *M* in 0.05 *M* phosphate buffer, pH 7.0. The excitation light for the CPL spectra was filtered by a broad band chemical filter (see Schlessinger et al., 1975.) Absorption anisotropy factor, $g_{ab}$, for azurin and apoazurin (calculated from the data of Tang and Coleman (1968) using the equation $g_{ab} = \Delta\epsilon/\epsilon$).

The spectrum of $g_{em}$ of apoazurin at pH 1.5 shows pronounced deviation from constancy. It is about threefold smaller at the red edge of the spectrum than at the blue edge. This behavior reflects heterogeneity in the population of the emitting chromophores and may be due, in part, to the preferential emission of the tyrosine and tryptophan residues in the different regions of the spectrum. The fact that the protein possesses appreciable rotatory power at pH 1.5 indicates that even in acid pH it still retains some tertiary structure. The fluorescence decay data (see Table I) show that at acid pH even the emission due to the tryptophan residues alone stems from a heterogeneous population of molecules. Thus, the decay of the fluorescence excited at 295 nm to prevent tyrosine excitation and collected at the wavelength range of 320–380 nm (using the filter Kodak 18A) is very clearly not monoexponential. An attempt to fit the fluorescence decay to a monoexponential function resulted in an unacceptable RMS of the residuals (Table I). The decay could be fitted to a biexponential decay with lifetimes of 4.1 and 1.1 nsec, and a ratio of amplitudes of about 1:5.

It may be noted that the fluorescence lifetime of NATA at pH 1.5 (in dilute HCl) is only 0.9 nsec. The lifetimes of the apoprotein at this pH are appreciably longer. Thus, the environment of the tryptophan of the protein at this pH is significantly different than that of NATA in water, provid-

[4] The monoexponential fluorescence decay observed for apoazurin does not exclude the possibility of heterogeneity of the kind in which a fraction of the molecules are not fluorescence at all. This possibility can in principle be checked by use of eq 7 if one assumes that the indole chromophore has the same $\tau_0$ in NATA and azurin. In the present case $\alpha_k = 1$, $\tau_k = 4.7$ nsec, $q_r$ (relative to NATA) = 1.5, and $\tau_r$ (of NATA) = 2.9 nsec. $F_{nf}$ calculated is negligible within experimental error. It should be noted, however, that due to the difference in the emission wavelength of apoazurin and NATA, the radiative lifetime, $\tau_0$, of the two may not be the same on theoretical grounds (Strickler and Berg, 1962), according to which $\tau_0$ is expected to be proportional to a weighted average of the emission wavelength cubed. It will therefore be approximately 30% lower for azurin than for NATA. If this is true a portion of the apoazurin molecules (about 30%) is fully quenched. The experimental results quoted (Weinryb and Steiner, 1968; Badley and Teale, 1969) do not support, however, a supposition of variation of $\tau_0$ of the indole chromophore.

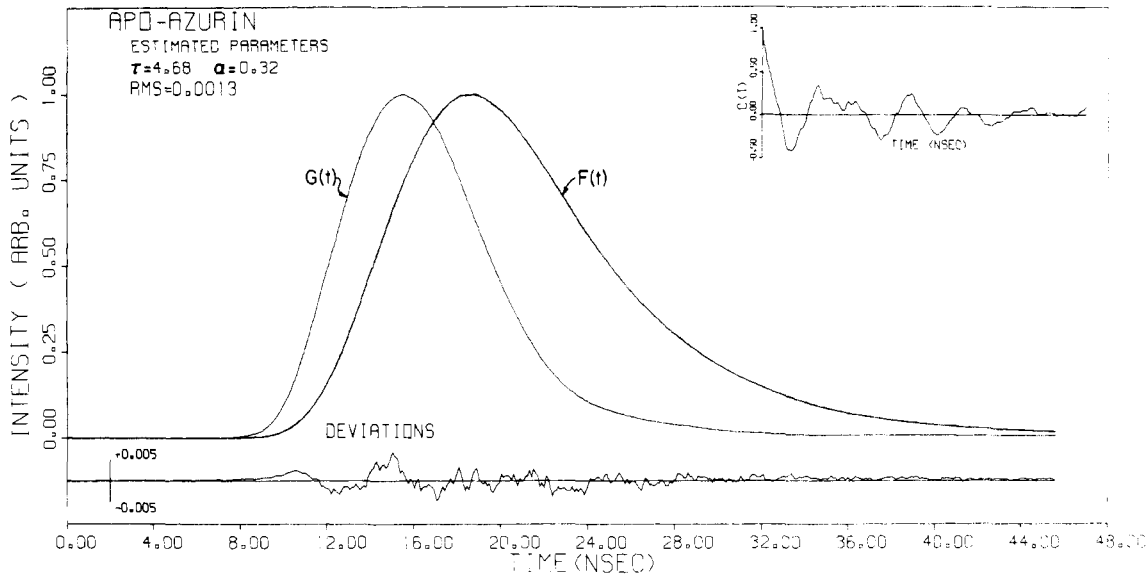


FIGURE 3: Analysis of the fluorescence decay of apoazurin assuming monoexponential function. Protein concentration, $2 \times 10^{-5}$; excitation wavelength, 280 nm; emission filter, Corning 7-60. Comparison with Figure 1, which shows the corresponding analysis for our lifetime standard, NATA, shows that the decay is monoexponential.

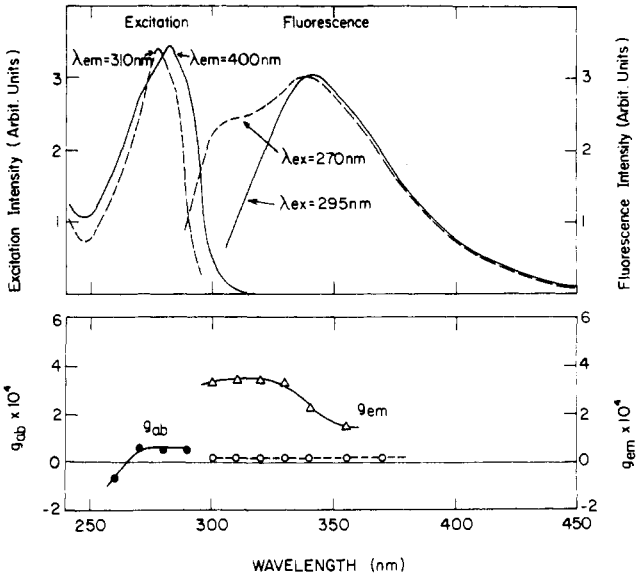


FIGURE 4: Spectroscopic data of azurin at pH 1.5 and in 6 *M* guanidine hydrochloride. Fluorescence spectra: of azurin in aqueous solution, pH 1.5; the excitation wavelengths are 270 or 295 nm as indicated; spectral resolution, 1.5 nm for both excitation and emission monochromators. Excitation spectrum of azurin in aqueous solution, pH 1.5; the emission wavelengths are 310 nm, at which tyrosine emission dominates, and 400 nm, at which tryptophan emission dominates; spectral resolution, 1.5 nm for both the excitation and emission monochromators. Emission anisotropy factor, $g_{em}$, of apoazurin in aqueous solution, pH 1.5 (Δ), and azurin in a solution of 6 *M* guanidine hydrochloride (O); protein concentration, $2.1 \times 10^{-4}$ *M*. The excitation light for the CPL spectra was filtered by a broad band chemical filter (see Schlessinger et al., 1975). Absorption anisotropy factor, $g_{ab}$, was calculated from the data of Tang and Coleman (1968) for native azurin, using the equation $g_{ab} = \Delta\epsilon/\epsilon$.

ing at least some screening from the aqueous environment.

*Denaturation by 6 M Guanidine Hydrochloride.* The emission anisotropy factor of apoazurin vanishes in 6 *M* guanidine hydrochloride (Figure 4). This indicates that on the average the environment exerts negligible asymmetric perturbation on the tryptophan in this denaturing solvent. It is significant that this happens even without disruption of the internal disulfide bond in the molecule, in contrast to other proteins which contain disulfide bonds, for which $g_{em}$ was found to vanish in 6 *M* guanidine hydrochloride only upon reductive splitting of the internal -S-S- bridges (Steinberg et al., 1974). Apparently, the environment of the tryptophan in azurin does not gain protection by the disulfide bond against disruption by the denaturant.

The fluorescence decay of apoazurin in 6 *M* guanidine hydrochloride is not monoexponential (Table I). Though it can be fitted to biexponential decay within experimental accuracy, it is probably composed of a spectrum of decay lifetimes. It may be noted, however, that the parameters obtained upon fitting to biexponential decay are similar to those obtained for other proteins under similar denaturing conditions (Grinvald et al., to be published).

*Effect of $D_2O$ and Pyridinium Ions.* Lakowicz and Weber (1973) have recently found that the tryptophan residue in azurin is accessible to quenching by dissolved molecular oxygen. We have similarly examined whether this residue, though buried in a hydrophobic environment, may be accessible to water molecules. On dissolution in $D_2O$ the emission and excitation spectra of apoazurin are the same as in $H_2O$; however, the quantum yield rises by 15% (±10%). In parallel, the lifetime is increased by 6 ± 1% (see Table I), the decay being monoexponential. To minimize possible errors in this experiment, a lyophilized fresh sample of apoazurin was divided into two portions; the first was dissolved in 0.1 *M* phosphate buffer (pH 7) in $H_2O$, and the second in 0.1 *M* phosphate buffer (pD 7) in $D_2O$. Lifetime measurements were performed for both solutions several times under identical conditions. For comparison, it is important to note that the quantum yield and lifetime of *N*-acetyl-L-tryptophanamide or *exposed* tryptophan residues in proteins are higher in $D_2O$ than in $H_2O$ by about 10% (Grinvald and Steinberg, 1974b). The change in the quantum yield and lifetime of apoazurin upon substitution of $H_2O$ by $D_2O$ is probably not due to a significant change in molecular conformation, since the CPL is the same in both solvents. This observation suggests that water molecules penetrate the hydrophobic environment of the tryptophan residue. Possibly, the mechanism of structural fluctuations

of the protein molecule previously proposed for quenching by molecular oxygen (Lakowicz and Weber, 1973), also operates in the case of the fluorescence enhancement by $D_2O$, permitting the exchange of entrapped water molecules or the exchange of hydrogen atoms in the vicinity of the tryptophan residue.

In contrast to $D_2O$, pyridinium ions at a concentration of 0.1 *M* seem to be inaccessible to the tryptophan residue. While these ions decrease the lifetime of NATA by about 85% under similar conditions, they have little effect on the average lifetime of apoazurin (Table I). Much larger quenching was observed with other proteins (Grinvald and Steinberg, 1974b). Slight deviation from monoexponential decay is, however, detected in the presence of 0.1 *M* pyridinium perchlorate, which probably reflects partial binding of the solute or change in conformation of some of the molecules.

*Native Azurin*

The spectra of fluorescence emission and florescence excitation of azurin at pH 7.0 are presented in Figure 2. These spectra are identical with the corresponding ones for apoazurin. Within experimental error, the excitation spectra for light emitted at 300, 310, and 360 nm are identical. Thus, if the two tyrosine residues in the molecule contribute significantly to the emission spectrum, they must have a fluorescence spectrum which is very similar in shape to that of the tryptophan fluorescence. Since this is not very likely, the emission band seems to be predominantly due to tryptophan emission, though very small tyrosine contribution cannot be excluded. The peak of the emission at 308 nm and the structured spectrum obtained point again to a highly hydrophobic environment for the tryptophan residue (Konev, 1967).

The CD and CPL spectra of azurin at neutral pH are presented in Figure 2. The values for $g_{ab}$ were calculated from the data of Tang and Coleman (1968). As in the case of apoazurin, the value of the absorption anisotropy factor, $g_{ab}$, varies markedly across the absorption region studied. This is due to the multitude of chromophores involved in the absorption in this region. In contrast, the value of $g_{em}$ is approximately constant across the emission band. Such behavior accompanies as a rule a homogeneous system (Steinberg, 1975; Steinberg et al., 1974); it is, however, no *proof* of homogeneity for a few reasons. A collection of dissimilar chromophores which happen to have identical emission spectra or identical values for $g_{em}$, or both, will also show a constant value of $g_{em}$ across the spectrum. Moreover, if the heterogeneity is due to the presence of a fraction of nonfluorescent molecules in the system, the observed $g_{em}$ may still be constant. That these situations indeed prevail in the case of native azurin was shown by the study of its fluorescence decay.

The fluorescence decay of native azurin at pH 7.0 is definitely not monoexponential (see Table I). It can be fit to a biexponential decay function with lifetimes 4.7 and 0.3 nsec, and a respective ratio of amplitudes of 1:2. The purity of holoazurin is proven by the monoexponential decay kinetics observed for apoazurin since the latter was prepared from the same holoazurin. Furthermore, the presence of apoprotein in the holoprotein solution can be excluded since addition of copper ions to the holoprotein solution did not further decrease the quantum yield, indicating that the holoazurin studied is 1:1 complex. (This is also evident from the $OD_{625}/OD_{280}$ ratio of 0.48 which was observed.) As was pointed out elsewhere (Hazan, 1973) the relative contribution to the total light intensity of a given term in a multiexponential decay is proportional to the product of the corresponding lifetime and the amplitude. Thus, the second exponential term which appears in the decay of azurin contributes about 10% of the total amount of light that is emitted by holoazurin $[\alpha_2\tau_2/(\alpha_1\tau_1 + \alpha_2\tau_2)]$. The possibility that the small second exponent is due to contribution of tyrosines to the total emission is excluded by exciting the protein solution at 295 nm, where tyrosine absorption is negligible. A second component of almost the same characteristics was detected also in this case (Table I).

The quantum yield of azurin is considerably lower than that of the apoprotein. Addition of an equivalent amount of $Cu^{2+}$ to a solution of apoazurin caused a drop in the fluorescence to 1/5.4 of its initial value. The excitation light was 295 nm; therefore only the tryptophan residue is involved in the above change. Upon conversion of apoazurin to azurin the extinction coefficient at 295 nm increased by about 20%, which may be due either to absorption by the copper or to an increase in the absorptivity of the tryptophan residue, e.g., by a small red shift in the spectrum. Thus, the quantum yield of azurin relative to the apoprotein, reckoned per light absorbed by the Trp residue, is 1/5.4 or 1/6.5, depending on whether the copper absorbs at 295 nm or not. This is about twice the value reported previously (Finazzi-Agrò et al., 1970). The reason for the discrepancy is not known. A comparison of the fluorescence decay data of apoazurin and holoazurin with their relative quantum yield forces the conclusion that a large fraction of the azurin molecules are completely nonfluorescent, thus demonstrating heterogeneity of the population of the protein molecules. Applying eq 7,[5] one obtains for the fraction of fully quenched molecules, $F_{nf}$, 53 or 62% depending whether the quantum yield of the holo- relative to the apoprotein is 1/5.4 or 1/6.5. Thus, *the majority of the native azurin molecules are fully quenched.* The fluorescence lifetime measurements on azurin thus contradict the explanation presented previously (Finazzi-Agrò et al., 1973) for the low quantum yield of holoazurin. According to that explanation the quenching of the tryptophan fluorescence in apoazurin upon binding of copper ions results from enhanced internal conversion to the ground state. If this were true, the lifetime should decrease in the same proportion as the quantum yield, which is definitely not the case (see Table I). Instead, ~60% of the molecules are fully quenched, as shown in the present study. The heterogeneity displayed by the tryptophan fluorescence may be due to differences in the mode of interaction of the $Cu^{2+}$ ions with the protein residues in the binding site; for example, by variations in the coordination of the copper in its binding site which reshuffle at a rate which is *slow* on the time scale of the lifetime. The tryptophan residue is either directly involved in this process, or is influenced by conformational changes accompanying the process. In this connection it is of interest to note that ligand exchange of divalent copper ions was indeed found to require about $10^{-8}$ sec (Eigen and Tamm, 1962; Poupko and Luz, 1972).

In the CPL spectrum of azurin, $g_{em}$ is seen to be significantly larger than for apoazurin (Figure 2). This difference indicates that the asymmetry of the environment of the

[5] The application of eq 7 requires a similar value for $\tau_0$ for the systems compared. This is most probably fulfilled in the present case in view of the identity of the fluorescence spectra of apo- and native azurin.

tryptophan residue in the fluorescent population of the azurin molecules is different from that of the tryptophan residues in apoazurin. This implies that the copper ion affects the tryptophan residue also in the fluorescent population of the protein molecules. Upon denaturation in 6 *M* guanidine hydrochloride, $g_{em}$ vanishes. This behavior is similar to that of apoazurin, but differs from that of other proteins with internal disulfide bonds (Steinberg et al., 1974), where disruption of these bonds was required for abolishing the CPL by the denaturant.

The decay kinetics of the emission of denatured holoazurin is identical within experimental error to that of denatured apoazurin (Table I). This is not surprising because copper ion is probably not bound in the denatured state (Finazzi-Agrò et al., 1970). The heterogeneity of the tryptophan environment also in the denatured state is disclosed by the different decay kinetics observed for the red and the blue regions of the fluorescence emission (Table I). It is of interest to note that the "red" tryptophans, which are presumably the more exposed ones, have a lifetime of 4.2 nsec, which is significantly longer than the lifetime of NATA in 6 *M* guanidine hydrochloride (2.9 nsec).

The fluorescence decay cannot be fitted to monoexponential decay also on reduction of the copper ion in azurin to the monovalent state. Upon fitting to biexponential decay, the lifetimes obtained are similar to those of the protein–$Cu^{2+}$ complex, i.e., 4.8 and 0.3 nsec (see Table I). The amplitude of the second term is, however, smaller in the reduced protein. The valence of the copper thus has some effect on the tryptophan fluorescence, possibly through differences in the mode of complexing to the protein at the metal binding site.

The existence of heterogeneity in the population of reduced azurin is also clearly manifested by the significant variations in $g_{em}$ values throughout the emission band (Figure 2). The interconversion of the two conformers of reduced azurin has also recently been observed by chemical relaxation studies of its electron exchange reaction with P-551 cytochrome *c* (Pecht and Rosen, 1973; Rosen and Pecht, to be published).

In conclusion it should be pointed out that the present study of the homogeneity and variability of azurin and its derivatives under various conditions is related in principle to a restricted region of the protein molecule, i.e., the tryptophan residue and its environment. By the study of the tryptophan fluorescence it is not possible to generalize directly to the other parts of the macromolecule, which may occupy in fact most of the molecular volume. Notwithstanding, the information derived by means of the tryptophan fluorescence is by no means trivial, and it might be of interest if other regions of the macromolecule could be investigated, e.g., by other probes.

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# Covalent Structure of Collagen: Amino Acid Sequence of α1-CB3 of Chick Skin Collagen†

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ABSTRACT: The amino acid sequence of α1-CB3, a peptide containing 149 residues obtained from the central portion of the α1(I) chain of chick skin collagen by cyanogen bromide cleavage, has been determined. As in the other sequences from the helical region of collagen chains, the repeating triplet Gly-X-Y extends throughout the length of the peptide. These data allow a comparison of the sequence to that of α1-CB3 from calf and rat skin collagens. As compared with calf, the chick peptide contains 14 amino acid differences, whereas it contains 17 residue differences from the rat peptide. Thus, the sequence identity level is 91 and 89%, respectively, in comparison to the calf and rat peptides. These values are significantly greater than the value of 97% observed between the peptide of the two mammalian species and reflect the greater phylogenetic distance of the species compared.

Knowledge of the primary structure of collagen is essential in elucidating the relation of structure to function. Information on the comparative biochemistry of collagens from different classes of vertebrates may, as has been shown in other proteins, significantly contribute toward our understanding of the aspects of primary structure which determine the biologic properties of the protein. In recent years, several laboratories have been committed to the determination of the complete covalent structure of several different collagens. Progress with the amino acid sequence analysis of this very large molecule, ca. 300,000 daltons, has been greatly facilitated by the application of CNBr cleavage (reviewed by Gallop et al., 1972; Traub and Piez, 1971).

As a part of our systematic effort to determine the complete amino acid sequence of chick skin collagen, we have previously reported the covalent structures of α1-CB1, α1-CB2, α2-CB1, and α2-CB2 peptides (Kang and Gross, 1970; Highberger et al., 1971). In the present communication, we describe our data delineating the amino acid sequence of α1-CB3, which contains 149 amino acids and which comprises residues 419 through 567 of the intact α1(I) chain. The covalent structure of the homologous peptides from calf skin (Fietzek et al., 1972a; Wendt et al., 1972a) and rat skin (Butler et al., 1974a) has recently been published.

## Materials and Methods

*Preparation of α1-CB3.* The CNBr peptide, α1-CB3, was prepared from the α1 chains of purified, salt-extracted skin collagen of lathyritic chicks. The procedural details and the criteria of purity were previously described (Kang et al., 1969a,b).

*Enzymatic Hydrolyses.* Digestion with trypsin (TPCK[1] treated, Worthington) was performed in 0.2 *M* Tris (pH 7.6) containing $10^{-3}$ *M* $CaCl_2$ at 37° for 2 hr. A 1:50 molar ratio of enzyme/substrate was used. The digestion was terminated by acidification with 2 *M* acetic acid and lyophilization. Digestion with chymotrypsin (three times recrystallized, Worthington) was performed in 0.2 *M* $NH_4HCO_3$ (pH 8.0) at room temperature for 2 hr. An enzyme/substrate molar ratio of 1:50 was used. The reaction was terminated by lyophilization or by separating the products by gel chromatography.

*Maleylation and Demaleylation.* In some experiments, the peptides were maleylated prior to trypsin digestion to confine the tryptic cleavage to the arginyl residues (Butler et al., 1969). Following tryptic c hydrolysis, the treated peptide was demaleylated by incubation in 0.2*M* pyridine acetate (pH 3.0) for 6 hr at 60°.

*Column Chromatography.* The initial fractionation of tryptic and chymotryptic peptides was carried out by molecular sieve chromatography on a column (2 × 120 cm) of Sephadex G-50 S (Pharmacia) equilibrated with 0.04 *M* sodium acetate (pH 4.8). Samples were applied in 2 ml of the buffer and the column was eluted with the same buffer at a flow rate of 18 ml/hr.

The peptides obtained from the molecular sieve chroma-

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[1] Abbreviations used are: CNBr, cyanogen bromide; TPCK, L-1-tosylamido-2-phenylethyl chloromethyl ketone; SPhNCS, 4-sulfophenyl isothiocyanate; PTH, phenylisothiohydantion; ANS, 2-amino-1,5-naphthalenedisulfonic acid; EDC, *N*-ethyl-*N'*-(3-dimethylaminopropyl)carbodiimide.