Modification of a specific tyrosine enables tracing of the end-to-end distance during apomyoglobin folding

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Abstract In order to follow the overall geometry of the apomyoglobin molecule during folding, we have converted a specific tyrosine residue into 3-nitro-tyrosine. The specificity of the modification was verified by proteolytic cleavage of the modified protein and mass spectroscopy of the resulting fragments. By measuring the energy transfer from the tryptophanyl side-chains to the modified residue the average end-to-end distance can be followed. The experiment shows that after initiation of folding the N- and C-termini are rapidly brought into proximity, possibly to a near-native distance.

Key words: Protein folding; Energy transfer; Nitro-tyrosine; Stopped-flow

1. Introduction

Our knowledge of the protein folding process has become increasingly detailed in recent years due to the development of novel techniques which are capable of probing the conformation of the polypeptide chain on the millisecond time-scale [1]. Among the most essential techniques are deuterium exchange pulse labelling [2,3] and the application of site-directed mutagenesis [4,5] which provide detailed local information about the secondary structure and the side-chain interactions, respectively. Other important methods are far UV CD which probes the average amount of secondary structure, near UV CD which probes the packing of the aromatic side-chains, and tryptophanyl fluorescence which provides information about the environment of this hydrophobic side-chain [6].

The results obtained by application of these techniques in all cases relates strictly to the local conformations, and conclusions about the global structure of the polypeptide chain during the folding process can only be indirectly inferred. Among the techniques that provide information about the overall geometry of a protein is fluorescence energy transfer [7]. This method exploits the fact that the emission of a fluorophore (the donor) can be quenched in the presence of another chromophore (the acceptor) if the fluorescence spectrum of the former overlaps with the absorption spectrum of the latter. The quenching is strongly distance dependent [8], and thus the observation of a reduced fluorescence intensity is an indication of proximity of the two groups. In an early work, Tsong applied the measurement of energy transfer from Trp to the heme group to the folding of ferricytochrome *c* [9]. More recently, Kawata and Hamegushi have followed the energy transfer from Trp to an AEDANS group during folding of the constant fragment of an immunoglobulin light chain [10]. The AEDANS group was attached to a free cysteine side-chain. Interestingly, the CD signal at 218 nm and conventional tryptophanyl fluorescence displayed identical kinetics for this system, but the energy transfer measurements revealed a much faster phase, indicating an initial collapse of the polypeptide chain into a compact conformation.

In the present work, we have applied this technique to the folding of apomyoglobin. Since the discovery of a stable intermediate at pH 4.0 on the acid denaturation curve [11,12] substantial experimental effort has been devoted to the study of this system [13]. The positions of the Tyr and Trp residues make horse heart apoMb well suited for study by energy transfer using the Trp-Tyr(NO_2) donor–acceptor pair [14]. The protein has 153 amino acid residues, with Trp residues at positions 7 and 14 and Tyr residues at positions 103 and 146 [15]. It is possible to modify Tyr^{146} specifically into Tyr(NO_2) (see below), and the measurement of Trp-Tyr(NO_2) energy transfer thus essentially characterizes the distance between the N- and C-termini. The Förster distance of the Trp-Tyr(NO_2) pair is about 26 Å [14]. In the native state of horse heart apomyoglobin the distance to Tyr^{146} is about 21 Å for both Trp side-chains [16], and since it is believed that native apoMb resembles native holoMb [17,18], a clearly visible effect of the presence of Tyr(NO_2) on the Trp fluorescence is expected for this state. New information about the geometry of the folding intermediate can then be obtained by monitoring the Trp fluorescence during folding of apoMb in a stopped-flow apparatus. In a parallel study, the energy transfer in the equilibrium intermediate has been investigated by measuring the time-resolved fluorescence decay curve with sub-nanosecond temporal resolution [19].

2. Materials and methods

2.1. Preparation of apoMb-Y146(NO_2)

Horse heart myoglobin was purchased from Sigma, and apoMb prepared using a modification [20] of the 2-butanone extraction procedure of Teale [21]. The product gave only a single band in 20% homogeneous SDS-PAGE. To convert Tyr^{146} into Tyr(NO_2) the protein was dissolved at 1 mg/ml in 40 mM phosphate buffer at pH 6.0. An aliquot of 75 μl of a 1% solution of tetranitromethane (TNM) in ethanol was added for each 850 μl of protein solution, and the reaction mixture was

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Abbreviations: CD, circular dichroism; AEDANS, 5-(((acetylamino)ethyl)amino) naphthalene-1-sulfonic acid; apoMb, apomyoglobin; Tyr(NO_2), 3-nitro-tyrosine; apoMb-Y146(NO_2), apoMb with Tyr^{146} converted to Tyr(NO_2); SDS-PAGE, sodium dodecyl sulfate polyacryl-amide gel electrophoresis; TNM, tetranitromethane; HPLC, high performance liquid chromatography; MALDI-TOF, matrix assisted laser desorption ionization – time-of-flight (mass spectroscopy); ACH, α-cyano-4-hydroxy-cinnamic acid; GuHCl, guanidine-hydrochloride.

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left with stirring in the dark. After 10 min another 75 μl of TNM-solution was added, and after 10 min of further reaction the solution was applied to NAP5 gel filtration columns (Pharmacia, Sweden) equilibrated with pure water. The protein containing fractions were subsequently eluted with water and collected.

The conversion of Tyr to Tyr(NO_2) using TNM is well known [22,23], and it has been shown that in some cases a single Tyr residue may be specifically modified [24]. Usually, the reaction is carried out at pH 8.0, but for apoMb it was revealed by SDS-PAGE that modification under these conditions is accompanied by partial degradation of the protein even for low concentrations of TNM (data not shown). The degradation may be due to the presence of trace amounts of proteases. Since the activity of many proteases is lower at pH 6.0 [25] the procedure described above was adopted instead. SDS-PAGE of the product obtained under these conditions showed no impurities.

In order to characterize the modified protein it was digested in 2 M urea by the glutamic acid-specific endoproteinase from *Bacillus licheniformis* [26]. The resulting peptides were separated by reverse-phase HPLC and analyzed by MALDI-TOF mass spectroscopy on a Finnigan Lasermat 2000. Fig. 1 shows the mass spectra of the Leu^{86}-Glu^{105} fragment containing Tyr^{103} and the Leu^{137}–Glu^{148} fragment containing Tyr^{146}. The ACH matrix used for optimal resolution in MALDI-TOF mass spectroscopy reduces Tyr(NO_2) to Tyr(NO) or Tyr(NH) [27]. The predicted mass of the unmodified Leu^{85}–Glu^{105} fragment is 2316 a.u., which is in good agreement with the value observed. The predicted masses for the Leu^{137}–Glu^{148} fragment are 1468 a.u. with unmodified Tyr, 1482 a.u. with Tyr(NH) and 1497 a.u. with Tyr(NO). It is seen that Tyr^{146} is specifically modified. This was confirmed by amino acid analysis of the fragments (data not shown). Mass spectra of the Trp^{7}–Glu^{18} fragment showed that modification of the Trp residues by TNM [28,29] had not taken place.

2.2. Equilibrium spectra

Absorption spectra were measured on a Perkin-Elmer Lambda 9 spectrophotometer, and CD measurements were carried out on a Jobin-Yvon auto-dichrograph Mark V. The buffers were 40 mM phosphate at pH above 6.0 and 25 mM citrate-phosphate at pH between 3.0 and 6.0. Values of pH below 3.0 were adjusted by addition of HCl. The ionic strength in all solutions was adjusted to 0.04 M by addition of NaCl. Steady-state fluorescence spectra were measured on a Perkin-Elmer LS 50B fluorometer with excitation at 280 nm. At this excitation wavelength the fluorescence of the remaining Tyr residue is negligible compared to that of the two Trp residues. All spectra were recorded at 25°C.

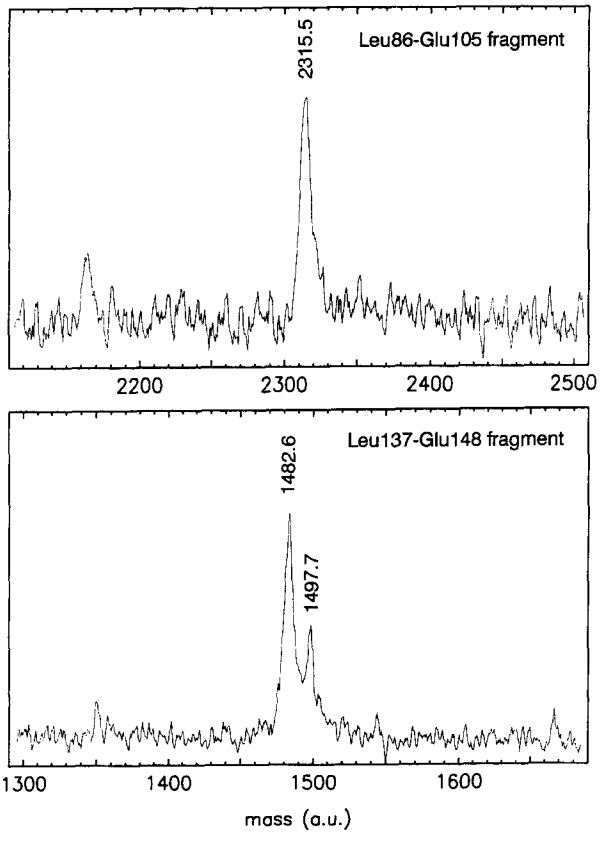

Fig. 1. Mass spectra of peptides prepared by proteolytic cleavage of modified apoMb. The spectra were calibrated with an internal standard of mass 1060 a.u.

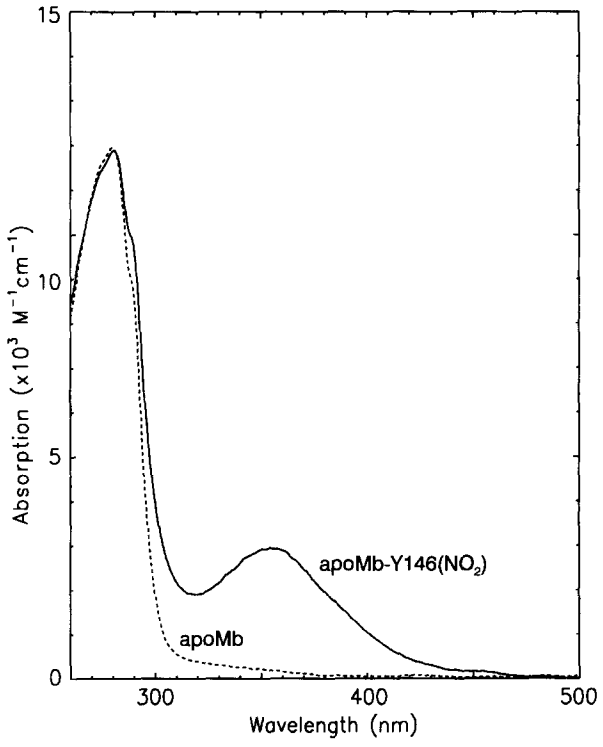

Fig. 2. Absorption spectra of apoMb and apoMb-Y146(NO_2) at pH 6.5. The peak at 280 nm is due to Trp and the peak at 360 nm is due to Tyr(NO_2).

2.3. Folding kinetics

Kinetic traces were recorded at 5°C using a Biologic SFM4 stopped-flow module. The protein was initially in denaturing conditions at pH 2.0 adjusted by addition of HCl, and folding was initiated by mixing the protein solution with 40 mM phosphate buffer at pH 6.5 in a ratio of 1:10. A 2 × 2 mm quartz cuvette was used for all experiments, giving a nominal dead-time of 5 ms. The light for excitation at 280 nm came from a Perkin-Elmer Lambda 9 spectrophotometer with the chopper disabled and was led to the cuvette through an optical fiber (Fiberguide Industries). The fluorescence was detected by a Hamamatsu R-376 photomultiplier connected to a Biologic PMS200 photomultiplier detection unit. A cut-off filter blocking transmission below 295 nm was inserted in front of the photomultiplier.

2.4. Förster distances

In the presence of an acceptor group, the fluorescence of the donor is quenched if the donor-acceptor distance is shorter than the Förster distance R_0 characterizing the donor–acceptor pair [8]. For a given value of the quenching Q an average distance r can be calculated from

$$r = R_0 \sqrt[6]{Q - 1}$$

Table 1
Trp-Tyr(NO_2) Förster distances for different states of apoMb

Conditions	pH 2.0	pH 4.0	pH 6.5
Förster distance	24.5 Å	26.0 Å	24.0 Å

We have calculated R_0 by a standard expression [7], using the value 0.14 for the quantum yield of the strongest fluorescent state [30] and normalizing the value for the other states relative to this. The results are listed in Table 1. The Förster distance is dependent on the relative orientations of the chromophores. Since these orientations are unknown, the value of R_0 can only be obtained with limited accuracy. A conservative estimate of the accuracy can be calculated from knowledge of the local dynamics of the chromophores, which one obtains from fluorescence anisotropy decay curves [31]. On the basis of measurements presented elsewhere [19] we have estimated the accuracy of R_0 to about ± 25%. The values of R_0 listed in Table 1 are based on average orientations.

3. Results

In Fig. 2 the absorption spectra of apoMb and apoMb-Y146(NO_2) are shown. The absorption peak of Tyr(NO_2) at 360 nm responsible for the quenching of Trp fluorescence is clearly visible. Fig. 3 shows the acid induced unfolding of unmodified and modified protein as monitored by CD at 222 nm. The curves are identical within the accuracy of the experiment and they are in good agreement with the results of Goto and Fink [32].

The Trp fluorescence spectra of horse heart apoMb as well as apoMb-Y146(NO_2) at pH 6.5 (native), 4.0 (molten globule) and 2.0 (denatured) are shown in Fig. 4. In the unmodified protein the fluorescence of the molten globule state is seen to be stronger than that of both the native and the denatured states. This agrees well with the results of Postnikova et al. [33] as well as with the properties of the GuHCl-induced equilibrium intermediate studied by Hargrove et al. [34]. Comparison with the spectra of the modified protein shows that while the fluorescence in the denatured state is unaffected by the presence of Tyr(NO_2), a substantial quenching is present in the native state as well as in the molten globule state.

Fig. 5 shows the Trp fluorescence during folding of apoMb and apoMb-Y146(NO_2). The horizontal lines show the fluorescence level obtained by mixing into denaturing conditions (pH 2.0). The decay curves were fitted to a sum of an exponential decay and a small linear drift, which gave decay times of 257 ± 6 ms for apoMb and 188 ± 44 ms for apoMb-Y146(NO_2). The fluorescence intensities relative to that of the denatured state at pH 2.0 are listed in Table 2 for the different states of the equilibrium and kinetic experiments. The values

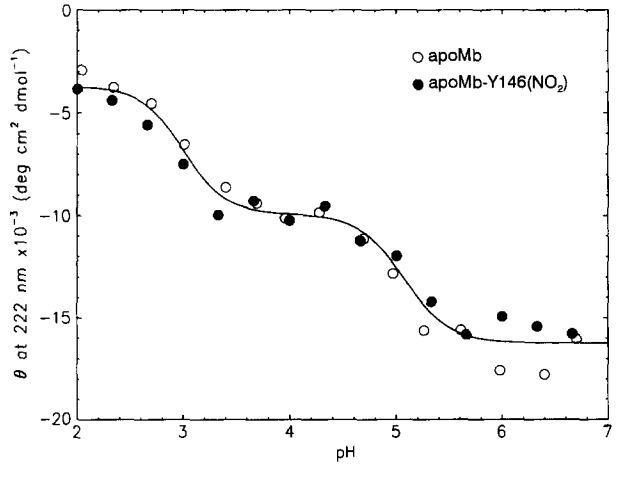

Fig. 3. Equilibrium acid-induced unfolding curve of apoMb (○) and apoMb-Y146(NO_2) (●), with conditions as described in the main text. The solid line is drawn to guide the eye.

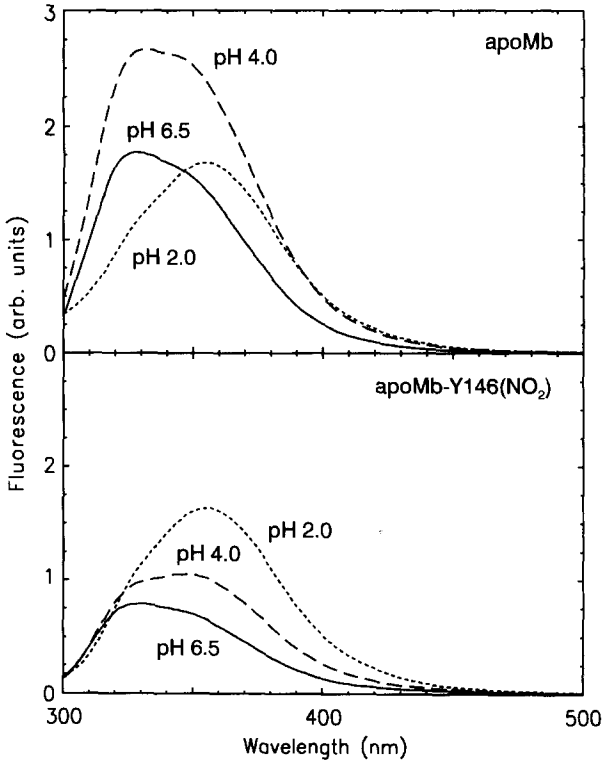

Fig. 4. Fluorescence spectra.

for the equilibrium states are found by integrating the fluorescence spectra, and the values for the kinetic states are taken from the fits, with the intensity in the folding products evaluated at 4 s after mixing. The background signal from pure buffer was subtracted in both cases.

4. Discussion

From the kinetic data shown in Fig. 5 it is clear that the presence of Tyr(NO_2) strongly affects the Trp fluorescence during folding. In particular, the fluorescence is quenched by a factor of 2.2 within the dead time of the stopped-flow apparatus, which shows that the N- and C-termini are rapidly brought into proximity (<5 ms). In their study of the folding of sperm whale apomyoglobin by deuterium exchange pulse labelling and far UV CD [35] Jennings and Wright have shown that a folding intermediate is rapidly established (<6 ms), with the A, G and H helices and part of the B helix formed. As these helices are amphipathic, it was proposed that they are stabilized by interactions in a single hydrophobic core. The present measurements lend strong support to this hypothesis. The earlier char-

Table 2
Fluorescence intensities relative to the denatured state

(A) Equilibrium	pH 2.0	pH 4.0	pH 6.5
apoMb	100%	149%	110%
apoMb-Y146(NO_2)	100%	67%	48%
(B) Kinetic	**pH 2.0**	**Intermediate**	**Product**
apoMb	100%	150%	105%
apoMb-Y146(NO_2)	100%	68%	57%

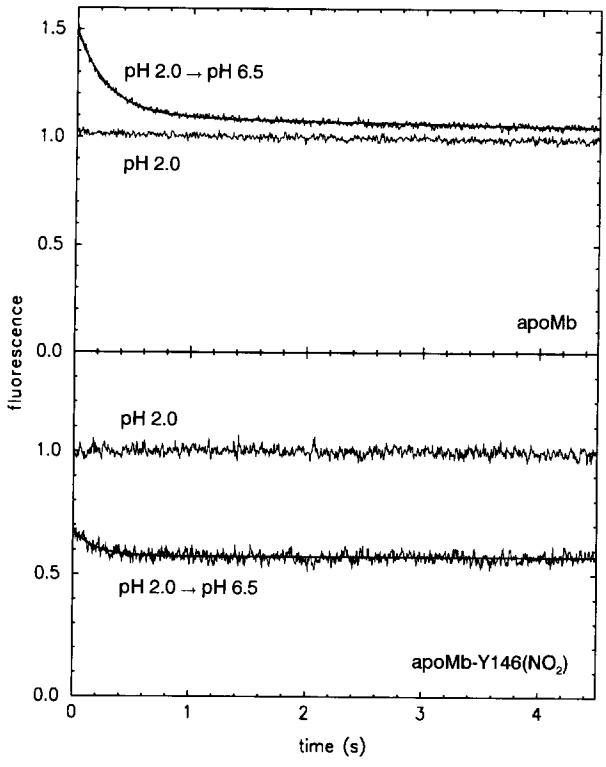

Fig. 5. Trp fluorescence intensity during folding obtained by mixing the protein solution at pH 2.0 (denaturing conditions) into renaturing conditions at pH 6.5. The bold lines show the fits.

acterization of the equilibrium intermediate had found the same helices to be formed [17], and very compelling evidence for identity between the two intermediates has thus been established. In the present work, the equilibrium experiments have been carried out at 25°C to facilitate comparison with the careful study of Goto and Fink [32], whereas the kinetic experiments like the work of Jennings and Wright [35] were performed at 5°C. Although this difference does not affect the stability at pH 6.5 [11], a strict quantitative agreement between the fluorescence intensities cannot be expected due to the temperature dependence of this parameter. However, the numbers listed in Table 2 establish a good qualitative agreement between the properties of the folding product and the native state, and between the folding and equilibrium intermediates.

The similarity of the acid denaturation curves shown in Fig. 3 indicates that the stability of the denatured, intermediate and native states are not significantly different in the unmodified and modified proteins. Application of the protein engineering analysis [5] to the folding times shows that the modification stabilizes the transition state for folding relative to the folding intermediate by 0.18 ± 0.14 kcal/mol. Tyr^{146} is not fully exposed to solvent in the X-ray structure [16] but these results imply that the addition of a nitro group does not cause any important changes of the local interactions. This suggests that the site of modification is in fact accessible to the solvent when the fluctuations of the structure are taken into account.

By using Eqn. 1, an average distance can be derived from the degree of quenching. From Table 2 it can be seen that the presence of Tyr(NO_2) quenches the Trp fluorescence by a factor of 2.2 in the folding intermediate and by a factor of 2.3 in the native state. By application of the Förster distances listed in Table 1 average distances of 25 Å for the intermediate and 23.5 Å for the native state are obtained. The closeness of these values suggests that the secondary structure elements observed in the folding intermediate by deuterium exchange pulse labelling [35] may be in a near-native tertiary geometry. This conformation is probably stabilized by non-specific hydrophobic helix–helix interactions, as it has been established that the specific native tertiary contacts are not formed in the equilibrium intermediate [36]. The distance obtained in the native state compares well with the 21 Å of the X-ray structure of myoglobin [16].

The present work demonstrates that fluorescence energy transfer can give detailed information about the geometry of folding intermediates. Trp and Tyr(NO_2) offers a simple donor–acceptor pair, which can be prepared by a gentle chemical modification. For proteins containing Trp residues and Tyr residues amenable to modification this method can be used to probe the collapse of the polypeptide chain. The exact nature of the information obtained will of course depend on the positions of the involved residues in the sequence of interest.

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