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CIRCULAR DICHROISM KINETICS OF ACID DENATURATION OF HEMOGLOBIN AND OF ITS β-SUBUNITS

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Human hemoglobin and its isolated β-subunits were denatured by addition of HCl so as to reach final pH values ranging from 2.0 to 3.2. The β-subunits were alkylated in both the β93 and β112 cysteines; this treatment makes the β-subunits monomeric. The kinetics of acid denaturation of the two proteins was followed spectropolarimetrically in the millisecond time range, measuring the changes in circular dichroism at 225 nm. At all pH values, in both systems, the decay of ellipticity could be simulated by two exponentials. The initial ellipticity values of the solutions, obtained by extrapolation at zero time, were those expected for the native proteins. The rates of denaturation were lower in the hemoglobin system than in the isolated monomeric β-subunits. The data suggest that in the tertiary structure of hemoglobin and βIAA there are different domains which unfold at different rates upon exposure to acid.

1. Introduction

The search of functional domains in hemoglobin, carried out in our laboratory for several years, has produced indications that in the system there are indeed domains smaller than a single subunit, whose librational motion is regulated by the presence of the heme and of ligands to the heme [1–6]. Similar evidence was obtained from experiments of denaturation at equilibrium using guanidine hydrochloride. Domains with different free energy of conformation could be detected and investigated in the isolated β-subunits of the protein. However, when acid was used as denaturant, it failed to show the presence of distinguishable domains [7]. The denaturation curves resembled very much the proton titration of a homogeneous group of ionizable residues, namely, the buried histidines of the system. Those experiments indicated that, if domains were present, they had a similar resistance to protein denaturation. This prompted us to investigate whether the various domains could by characterized by different rates of unfolding.

The availability in our laboratory of a spectropolarimeter with a stopped-flow attachement and a computerized signal averager allowed us to follow the change in time of the circular dichroism, in the far-ultraviolet region, of either hemoglobin or of βIAA (hemoglobin β-subunits alkylated at the β93 and β112 cysteines) upon exposure to HCl. In this way we could directly monitor conformational changes of the protein during the unfolding process. The data showed that the denaturation was not homogeneous in the sense that under all conditions tested the pseudo-first-order kinetics could be simulated by multiple exponentials, without detectable autocatalytic behavior. This would indicate the presence of several structural domains in the system, whose stabilities are not interdependent.

2. Materials and methods

Human hemoglobin and isolated β-subunits, carboxyamidomethylated at both the β93 and β112 cysteines (βIAA), were prepared as previously described [8]. The βIAA so obtained are monomeric [8]. Recycling through a mixed-bed resin column was used for removing organic and inorganic phosphates from the stock solutions of hemoglobins.

Protein concentration was measured spectrophotometrically using $\epsilon = 14000\ cm^{-1}\ M^{-1}$ for the carbonmonoxy derivatives at 540 nm.

For pH measurements a Radiometer M26 instrument was used equipped with combination electrodes obtained either from Radiometer or from Methrom.

The change in the millisecond time range of the optical activity of the various solutions was followed using a modified Jasco-20 spectropolarimeter equipped with a Jasco stopped-flow apparatus.

The spectropolarimeter was modified as follows by Mr. Jack Landis (Oakland H.J. 07436, 12 Deer Lawn Court). The Pockells' cell was removed, and a quarter wave plate was installed acoustically modulated with a nominal frequency of 50.3 kHz by a single crystal. The piezo-elastic modulator received programmed feed-back signals from the monochromator. In this way circularly polarized light was obtained by quarter wave delays at all wavelengths. A reference signal from the piezo-elastic modulator was sent to a phase detector. The light intensity after absorption by the sample was detected by a Hammamatsu R-376 phototube. The preamplifier of the phototube split the signal into d.c. and a.c. components. The d.c. component was used to maintain constant gain at the phototube. The a.c. component was sent to the phase detector and compared to the reference signal. The difference between the two was sent to the external recording device.

The spectrometer was connected to a MESA 3220 signal averager (Maine Scientific Associates, Bangor, ME). This instrument was based on a Motorola MC68000, 32 bit, microprocessor. The hardware also included a dual-channel 12 bit 333 kHz digitizer, 64 kbytes of RAM and 32 kbytes of ROM. A built-in CRT allowed graphic representations of the data. The large size (32 bits) of the words allowed true summation of the transients, without renormalization. Data were collected in blocks of up to 8000 words and either sent to a Morrow MD3 microcomputer for local data storage on disk, or via telephone to a mainframe computer, where the data were both stored on tapes and analyzed. Data analyses were performed using standard software developed in our laboratory, based on the nonlinear least-squares procedure developed by Marquardt [9], and on the PLOT10 graphic package of Tektronix.

The instrument was calibrated with standards of camphorsulfonic acid.

Kinetic measurements were performed using 0.07 mg/ml protein solutions in 0.1 M NaCl. In the stopped-flow apparatus these solutions were mixed with an equal volume of HCl in 0.1 M NaCl. The HCl concentration was adjusted so as to produce the desired final pH after mixing with the protein. A 1 cm path length cuvette was used for the observation.

All measurements were performed at 10°C. The carbonmonoxy derivatives of hemoglobin and βIAA were used. Before use all solutions were equilibrated with carbon monoxide at atmospheric pressure.

Total transient times ranged from 2.0 to 20 s. The time constant of the instrument was kept at least 5-times shorter than the half-times of the reactions.

The instrument per se was capable of a dead time of 10 ms. However, in these experiments, in order to avoid misinterpretations of the data (as discussed later), analyses of the transient started at 100–150 ms after mixing.

3. Results

In all cases exposure to acid produced a decay of ellipticity. This was analyzed assuming a pseudo-first-order reaction using a multiexponential model as in

$$t = ([\theta]_0 - [\theta]_\infty) \sum_i f_i \exp(-k_i t)$$

in which $[\theta]_0$ is the initial ellipticity of the samples

before exposure to acid, $[\theta]_\infty$ the final ellipticity of the samples after completion of the denaturation, f the fraction of the i-th component, and k the rate of the process. The value of $[\theta]_\infty$ was the average ellipticity of the samples over a 1 min interval, 5 min after mixing. This value remained constant for the next several minutes. The value of $[\theta]_0$ was obtained from the preexponentials of the various components.

In all the analyses, when single exponentials were used, simulations of the data were poor with respect to both visual inspection and statistics, showing large non-random distribution of the residuals. When two exponentials were used, the simulations became visually good as shown in fig. 1, with random distributions of the residues, and a 20–50-fold improvement of their mean square value. When three exponentials were used the resulting simulations had one component with either an amplitude near zero or a rate similar to that of another component, with no modification of the statistics of the residuals. Therefore the data presented in this paper represent two-exponential simulations. Fig. 1 shows a typical experiment in which 60 transients were accumulated for the denaturation of hemoglobin at pH 2.4. Table 1 summarizes the data obtained for hemoglobin and βIAA.

The value of the initial ellipticity, $[\theta]_0$, was in all cases $22.6 \pm 2.4 \times 10^3$ cm/dmol corresponding to the ellipticity of native hemoglobin and of native βIAA. This indicates that additional kinetic components were absent during the initial blind period of 100–150 ns.

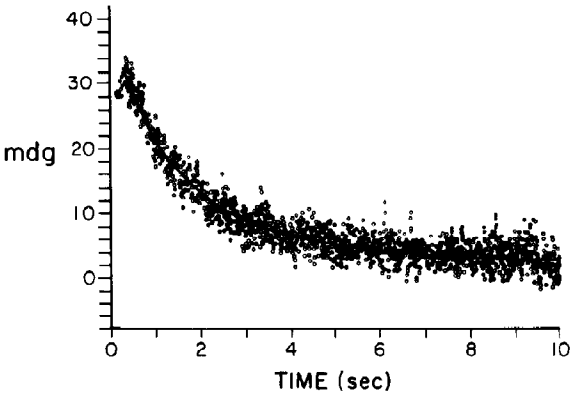

Fig. 1. Denaturation at pH 2.4 of carbonmonoxyhemoglobin followed at 225 nm. The experimental points are the average of 60 transients collected using a time constant of 16 ms. The continuous line was obtained using eq. 1 where $k_1 = 0.95$ s^{-1}, $f_1 = 0.62$ and $k_2 = 0.158$ s^{-1}.

4. Discussion

Luchins and Beychok [11] have reported stopped-flow CD data for the acid denaturation of methemoglobin. Their results were obtained with an instrument similar to ours, however, they did not mention detailed numerical simulation of the data and only $t_{1/2}$ values are reported. Those values are consistent with the $t_{1/2}$ values listed in table 1, computed from the kinetic constants obtained from the simulations. As mentioned above, two exponentials were necessary in order to justify our data, indicating the absence of cooperative behavior in the kinetics. Third components did not

Table 1

Pseudo-first-order kinetic constants obtained using eq. 1 from the ellipticity decay of hemoglobin and βIAA exposed to HCl

The % column indicates the relative amount of total decay corresponding to k_1. The half-time of the reaction was calculated using the listed values of k_1 and k_2.

Protein	Final pH	k_1 (s^{-1})	%	k_2 (s^{-1})	$t_{1/2}$ (s)
Hemoglobin	1.9	2.67 ± 0.22	68 ± 06	0.14 ± 0.11	0.16
	2.4	1.03 ± 0.13	59 ± 10	0.15 ± 0.03	0.53
	2.8	0.73 ± 0.37	58 ± 18	0.16 ± 0.11	0.67
	3.2	0.08 ± 0.01	85 ± 03	0.01 ± 0.01	6.00
βIAA	2.0	5.86 ± 0.70	73 ± 10	0.22 ± 0.12	0.08
	2.4	2.51 ± 0.26	63 ± 04	0.04 ± 0.02	0.28

seem to have statistical relevance. However, it is still possible that the two kinetic constants used in the simulations represent averages of two groups of similar kinetics whose individual components could not be resolved. It is interesting to observe that the relative amplitudes of the two kinetic constants were similar in both hemoglobin and βIAA, except at pH 3.2 in hemoglobin, where the system could have been in a region of at least partial reversibility.

The values of $[\theta]_{MRW}$ recorded 5 min after mixing were at all pH values $[\theta]_{MRW} = 7.0 \pm 0.5 \times 10^3$ for βIAA and $10.04 \pm 0.8 \times 10^3$ for hemoglobin. It should be stressed that these ellipticities are considerably higher than those obtained by Franchi et al. [7] under equilibrium conditions, with samples equilibrated for 3 h at the various pH values. The larger amount of secondary structure remaining 5 min after mixing suggests the presence of other kinetic components too slow to be detected on the time scale used in our experiments.

These considerations indicate that the acid denaturation is a complex event involving various structural domains each characterized by their own rate. Whether these domains represent long-range interactions diffused throughout the whole protein matrix or whether they represent specific regions of the molecule remains to be seen.

Rashin [12] has described the presence of two structural domains in myoglobin and in the isolated subunits of hemoglobin, which may support the hypothesis of localized domains. Also, Franchi et al. [7] have demonstrated, with denaturation equilibrium experiments using guanidine hydrochloride, the presence in βIAA of a heme-dependent domain of secondary structure. This domain can be reversibly unfolded at low guanidine hydrochloride concentrations before the destruction of the heme pocket and extraction of the heme. Although the approaches are different, it is tempting to equate the low-stability domain of Franchi et al. [7] with the fast kinetic component reported here.

Admittedly these are speculations, nevertheless, these data contribute to the increasing accumulation of evidence that hemoglobin subunits include structural domains with distinct characteristics of hydrophobicity [11], stability to guanidine hydrochloride [7], librational motions [1–6], and now of denaturation rate when exposed to acid.

The βIAA subunits at the concentration used in our experiments were in monomeric form [8], while the hemoglobin solutions contained dimers and tetramers. The very similar behavior of hemoglobin and βIAA suggests that the heterogeneity of the kinetics of acid denaturation in hemoglobin reflected an intrinsic characteristic of the tertiary structure of the subunits. The lower k_1 values were probably due to the presence of quaternary structure and in hemoglobin were half those of βIAA, producing longer half-times.

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