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TIME-RESOLVED SMALL-ANGLE X-RAY SCATTERING EXPERIMENT ON ASSOCIATION OF ISOLATED α- AND β-CHAINS OF HUMAN HEMOGLOBIN

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The time course of the recombination of hemoglobin from isolated α- and β-chains (human) was studied by following the change in small-angle scattering from a reaction mixture using synchrotron radiation. The scattering patterns were recorded successively within 51 s at intervals of 200 ms. The results suggest that when 2 mM solutions of CO-liganded α- and β-chains are mixed in stoichiometric amounts at pH 7.4, the tetramerization process is a pseudo-first-order reaction with a rate constant of 0.059 s^{-1}, i.e., a half-time of approx. 12 s.

1. Introduction

When α- and β-subunits of hemoglobin are mixed in stoichiometric amounts, the subunits associate spontaneously to form hemoglobin tetramers. This reconstituted tetramer has all characteristic structural and functional properties of native hemoglobin [1]. As the interaction of α- and β-chains is closely associated with the functional properties of the protein the kinetics of their recombination has received considerable interest. In the case of the deoxygenated derivatives, the formation of tetramers has been studied mostly by taking advantage of the spectral differences between hemoglobin and its isolated subunits [2–4]. However, for the ligand-bound form, such study is complicated by the absence of spectral differences between the isolated chains and hemoglobin [1].

Small-angle X-ray scattering can, in principle, give structural information about heterogeneous systems in solution. As first demonstrated by Moody et al. [5], the combination of synchrotron radiation and stopped-flow techniques makes it possible to follow the change of the 'structure' of the solution during assembly processes.

We describe here the results of a time-resolved small-angle X-ray scattering experiment on the time course of the tetramer formation process of CO-liganded α- and β-chains.

2. Materials and methods

2.1. Preparation of hemoglobin subunits

Human hemoglobin obtained commercially (Sigma) was used as starting material. The separation of α- and β-chains was done by the method of Bucci and Fronticelli [6], and the subsequent preparations of α^{SH}- and β^{SH}-chains from α^{PMB}- and β^{PMB}-chains according to the procedures of Yip et al. [7]. Each chain was finally collected in 0.1 M potassium phosphate buffer at pH 7.4. The yields

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Abbreviation: PMB, *p*-mercuribenzoate.

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of α- and β-chains were 15–20% in both cases. The solutions of isolated subunits were kept under CO atmosphere at 4°C before use.

2.2. Stopped-flow

The rapid mixing of isolated α- and β-chains was done with the stopped-flow apparatus designed by Moody [5] at the European Molecular Biology Laboratory. Equal volumes, each of 200 μl per shot, of α- and β-chain solutions (2 mM, pH 7.4) were mixed at 22–23°C. A modified version of the original apparatus was used in which measuring capillary had been replaced by a cell with mica window (5×8 mm^2) with an X-ray path length of 1 mm.

2.3. X-ray scattering

The scattering measurements were carried out on a double-focusing X-ray camera (X13) [8,9] at the EMBL Outstation on the storage ring DORIS of the Deutsches Elektronen-Synchrotron (DESY) at Hamburg (F.R.G.). During the experiments the accelerator was running at 3.3 GeV with an average circulating current of 60 mA. The scattering patterns were recorded using a linear position-sensitive detector [10] flushed with argon/CO_2 (66% Ar). The wavelength used was 0.148 nm, and the sample-to-detector length 2250 mm. For each stopped-flow shot, 256 spectra of 256 resolving points each were recorded at intervals of 200 ms. Data were accumulated in groups of 10–40 shots and stored on a disk. The scattering patterns were corrected for the intensity of the primary beam.

3. Results and discussion

After about 200 stopped-flow shots, five data sets were available. The resulting scattering patterns for each time slot (not shown) were too noisy, even after averaging, to be analysed individually. However, it was obvious from qualitative comparison between them that the slope of the scattering curve near the origin obtained at the initial state of reaction is smaller than that at the final state, confirming that association between the different subunits took place as a result of the reaction between the two solutions.

In order to follow the reaction process, we used the integrated scattering intensity, Q, which is defined as

$$Q = \int I(h)\mathrm{d}h$$

where $h = 4\pi(\sin\theta)/\lambda$, 2θ = scattering angle and λ = wavelength. The integration of intensity, $I(h)$, was done over the range of $1.3 \times 10^{-2} < h < 9.5 \times 10^{-2}$ Å^{-1} without subtracting the background scattering. The background scattering arising mainly from solvent is invariant throughout the reaction, and hence the integral of its intensity; in

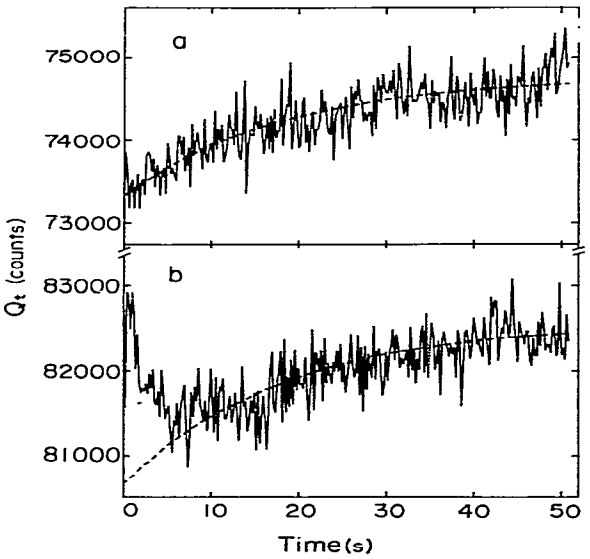

Fig. 1. Time courses of the changes in the integrated intensity Q at small angles on mixing the isolated α- and β-chains in 0.1 M potassium phosphate, pH 7.4. Q_t is Q at time t. Initial concentration of each chain = 2×10^{-3} M in heme. Time resolution = 200 ms. (a) Scattering pattern accumulated through 35 shots; (b) sum of (10 + 15 + 20) shots. Dotted lines indicate the simulated curves based on the mechanism illustrated in fig. 3. In calculations, both α- and β-chains were regarded as solid spheres of 17 Å radius, and the dimers (α_2, $\alpha\beta$) were represented by two spheres in contact and the tetramers (β_4, $\alpha_2\beta_2$) by four spheres in the symmetry of the tetrahedron in contact. Values of the kinetic parameters used are given in the text.

the case of hemoglobin solutions of 2 mM in heme, the background occupied about 75% of the total intensity within the range described above. This treatment resulted in two types of curves as illustrated in fig. 1. The exact cause of the difference between the two traces is not known but the initial decrease of Q in fig. 1b is most probably due to a mixing artefact. The data set which shows this type of behavior was obtained from less than 20 shots, whereas the curve in fig. 1a, which is more reliable, was obtained from 35 shots. Except for the first 10 s the two curves can be approximated by an exponential function, suggesting a first order for the overall reaction.

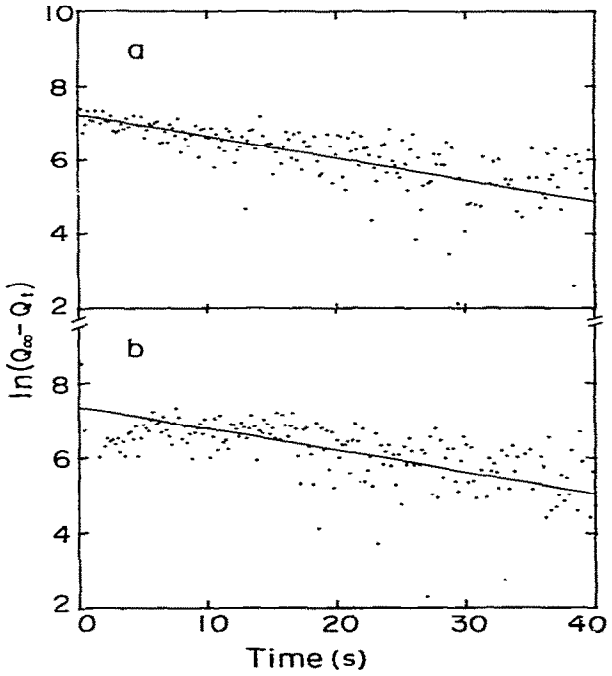

Fig. 2. Semilogarithmic plots of the time courses of the changes shown in fig. 1. Q_∞ on the ordinate is the value of Q_t at the final state of reaction, but is here substituted by the average of Q_t for the last 10 s. The regression lines were obtained by the least-squares method for the data points of $10 \leqq t \leqq 40$ s. The slope gives the rate constant, τ. The value of τ is slightly dependent on the choice of the time range to obtain Q_∞ or to be treated. (a) Data of fig. 1a, $\tau = 0.059\ s^{-1}$; (b) data of fig. 1b, $\tau = 0.058\ s^{-1}$.

Fig. 2 shows the corresponding semilogarithmic plots as well as the results of a least-squares curve fitting. From this analysis of the data in the range $10 \leqq t \leqq 40$ s, a rate constant of $0.059\ s^{-1}$ corresponding to a half-time of approx. 12 s was found. Fig. 3 illustrates one possible mechanism [11,12] for the tetramer formation process of isolated α- and β-chains. A computer simulation of the reaction based on this mechanism gave a best agreement when the rate constant ($0.059\ s^{-1}$) was used for k_2 of the $\beta_4 \rightarrow 4\beta$ dissociation (fig. 1). The values of the other parameters used were: $k_1 = 10\ s^{-1}$, $k_{-1} = 10^5\ M^{-1}\ s^{-1}$, $k_{-2} = 10^{15}\ M^{-1}\ s^{-1}$, $k_3 = 5 \times 10^5\ M^{-1}\ s^{-1}$, $k_4 = 10^6\ M^{-1}\ s^{-1}$ and $k_{-4} = 2\ s^{-1}$. These six parameters were made to vary around the reported values [4,11–14] with little influence on the rate constant. Furthermore, the simulation indicated that the reaction mixture contains a high proportion of β_4 and $\alpha_2\beta_2$ tetramers and α_2 dimers but that the contribution of other types of particles is small as shown by fig. 4.

The scattering pattern of a reaction mixture is a linear combination of the patterns of the initial and final states if there are no intermediate steps, as discussed by Moody et al. [5]. In the present case, however, the reaction is known to proceed via several intermediate molecular species and a simple interpretation is not applicable. The fact that the reaction, as observed by the change in Q, follows pseudo-first order kinetics suggests that it is controlled by a single rate-determining step, which the calculations suggest to be the dissociation of the β_4 tetramers – a picture which is consistent with the results of Rollema et al. [13], except for a difference in the value of the rate constant. These authors obtained a half-time of 4 s for the recombination reaction of CO-liganded α-

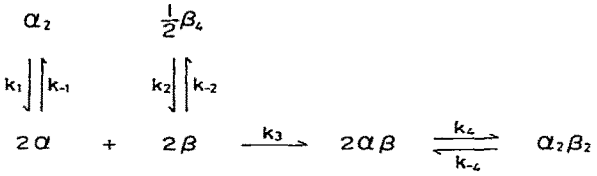

Fig. 3. mechanism of the formation of $\alpha_2\beta_2$ tetramers from isolated α- and β-chains of hemoglobin.

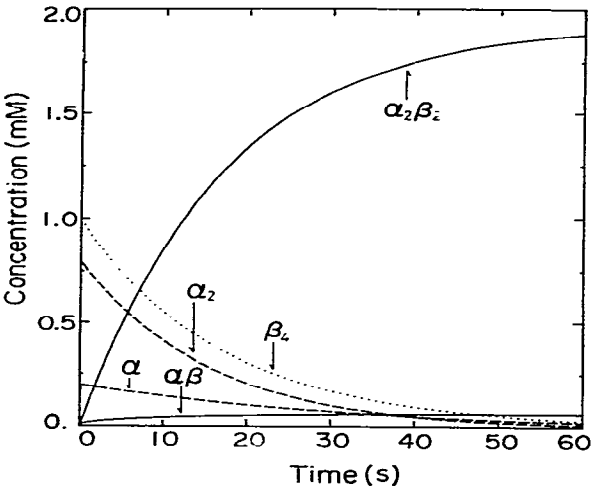

Fig. 4. Computer solution to the mechanism illustrated in fig. 3 using the values of the parameters described in the text. Concentration is expressed in heme. Concentration of β monomer is less than 0.6×10^{-6} M throughout the reaction (curve not depicted).

and β-subunits at 0.5 mM heme, pH 7.0, from measurements of pH changes whereas Antonini and Brunori [14], using flow-flash techniques, reported a half-time of 60 s at 0.5 M, pH 7.0. The differences between these observations and ours ($t_{1/2} = 12$ s) may be attributed to differences in the experimental conditions as well as in the method used to monitor the reaction. The influence of the experimental conditions on the value of the rate constant, which should lead to a more definitive interpretation, requires further experiments.

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