Biochimica et Biophysica Acta, 705 (1982) 257–263
Elsevier Biomedical Press

BBA 31238

RECOMBINATION KINETICS FOLLOWING NANOSECOND LASER PHOTOLYSIS OF CARBONMONOXYHAEMOGLOBIN

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(Received April 8th, 1982)

Key words: Recombination kinetics; Laser photolysis; Carboxyhemoglobin; Hemoglobin structure

The kinetics of the ultrafast ligand recombination following 347 nm laser photolysis of aqueous solutions of carbonmonoxyhaemoglobin have been investigated. The process is biphasic and the rate constants for the two processes as functions of temperature have been used to give activation energies of 6 ± 3.9 kJ·mol^{-1} for the fast process and 31 ± 4.8 kJ·mol^{-1} for the slow process. Frequency factors have also been calculated. The two processes are discussed in relation to both low-temperature studies and model calculations on the rate of entry of carbon monoxide into haem proteins.

Introduction

Nanosecond photolysis studies of carbonmonoxyhaemoglobin (HbCO) were first reported by Alpert et al. [1,2]. The results were interpreted on the basis of protein conformational changes. Subsequent investigations have conclusively shown that these absorbance changes on the nanosecond timescale are solely due to ligand recombination [3–5]. In the original work it was stated that the process followed first-order kinetics [2]. Subsequently, the same group have claimed that an $\exp(-kt^{1/2})$ rate law is applicable [6]. In a Raman study of the same process the analysis was based on first-order kinetics [7].

Frauenfelder and co-workers [8–10] have investigated recombination at low temperatures for single-chain haem proteins using microsecond flash photolysis. They found step-wise recombination processes. Extrapolation of their data indicated that the fastest recombination they observed corresponded to nanosecond timescale recombination at ambient temperature. At low temperatures the kinetics were non-exponential, but above approx. 200 K normal exponential rate laws were observed. The rates of the fast recombinations were independent of CO concentration. The rates of recombination to the α and β chains predicted at ambient temperature were sufficiently different for (at least) biphasic kinetics to be expected in nanosecond laser photolysis studies.

The present investigation by non-linear least-squares analysis of the kinetics of recombination of CO to haemoglobin was carried out in an attempt to resolve the contradictions in these findings.

Materials and Methods

Solutions of human carbonmonoxyhaemoglobin of, typically, 50 μM haem concentration in 0.1M potassium phosphate (pH 7.0) buffer were prepared and photolyzed with 30 ns half-width, 347 nm pulses as described previously [4]. The oscilloscope traces were photographed at approximately $\times 1$ magnification and digitized directly from the photographs using an adapted XY recorder. This has a manually operated magnifying eye-piece and was directly connected to a Prime 400 computer. Data files containing about 100 points each were transferred to the CDC 7600 computer at the University of Manchester Regional Computing Centre where the non-linear

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0167-4838/82/0000–0000/$02.75

least-squares fitting program is stored. The program is a substantially extended and modified version (Catterall, R. and Duddell, D.A., unpublished data) of that described by Dye and Nicely [11].

In the present study there are three areas in which the kinetic model is approximate and could lead to systematic error in the analysis. The first concerns the pulse-width of the excitation. The effect of this on kinetic studies has been quantified on the basis of synthesized data [12]. The half-life of the fast component in these decays exceeds the pulse-width for all but the highest-temperature experiment and the analyses have been based on data collected from a point just below the peak of each kinetic trace. Thus the values obtained for the fast-component rate constants will be systematically low because of the neglect of the convolution of the decay with the excitation pulse function, but with an error substantially less than 5%.

The second possible systematic error is in the neglect of conformational changes in the protein that are taking place concurrently with the ligand recombination. It should first be noted here that it has been claimed that the interpretation of the original nanosecond laser studies, which used a 30 ns pulse [1,2], was incorrect because "the spectral evolution of the free Hb and the ns recombination partially overlap each other", thus "it was not possible to separate the two processes" [5]. This claim cannot be substantiated, however, because the spectra obtained with a 30 ns pulse, which were used to demonstrate that recombination was occurring [4], are identical to those subsequently obtained with a 7 ns pulse [13]. Nonetheless, the kinetic analysis could be affected by this partial overlap. Fortunately, however, the wavelengths used for observation in this work, 416 nm and 438 nm, which show maximum absorbance change during recombination, are two at which Hb and Hb* are essentially isosbestic. Therefore the kinetic analysis of the recombination will not be in error because the conformational change is ignored.

Finally, there will be an error if the different stages of recombination are treated separately when in reality they overlap. Fig. 1 shows a trace obtained over an extended timescale of some 5 μs. It shows that after approx. 3 μs, when the initial absorbance change is more than 99% complete, the trace approximates well to a horizontal line. Thus, recombination at slower rates is insignificant over the timescale of the analysis of the nanosecond recombination.

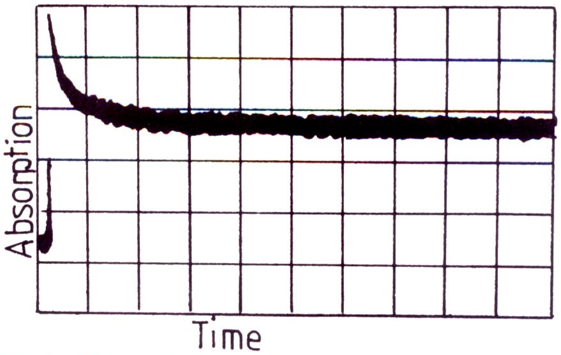

Fig. 1. 500 ns/division oscilloscope trace for photolysis of aqueous HbCO at 278 K with analysis wavelength 438 nm.

A vital aspect of non-linear squares analysis is the weighting of the data in accordance with the errors in the measurements [14]. Since the signal measured from a photomultiplier is dependent on the number of photons reaching the detector, there will be a Poisson error associated with the voltage measured. In this case the weight given to each y-coordinate measurement should be independently determined from the value of the y-coordinate itself [15]. However, in these experiments, where the transmission of the sample was measured, small changes in a relatively high photon count were detected and so the weights for each y-coordinate can be considered to be the same. That this approximation is a reasonable one can be seen from the residual plot in Fig. 6. This was obtained for a model that fits the data well. It does not show the wedge-shape that would be expected if the weights were dependent on the value of the y-coordinate.

Reasonable estimates of the errors have been made which reflected the relative inaccuracy of the y-coordinates, due to the noise on the data, with respect to that of the x-coordinates. Because the distribution of these errors was not determined experimentally, the value of the reduced chi-square statistic obtained by the fitting process cannot itself be used as a measure of goodness-of-fit. However, the values obtained for the same set of data fitted with different models can be compared by means of an F-test [15].

Results

Because the extent of nanosecond recombination increases with decreasing temperature, the best kinetic traces were obtained at the lowest temperature studied, 278 K. Fig. 2 shows a typical example. A suitably sensitive vertical scale was chosen for this so that the decay essentially filled the oscilloscope screen. The appropriate value of I_0 and of the final level of absorption for the analysis of this trace were calculated by comparison with a trace such as that shown in Fig. 1. This was obtained under identical experimental conditions but with different oscilloscope settings.

Analysis of this particular trace as a single-exponential decay gave a value of $k = 3.50 \cdot 10^6\ \mathrm{s}^{-1}$. The value of the reduced chi-square statistic was 1.9 (for 135 degress of freedom). The plot of the residuals for this analysis is shown in Fig. 3. It has been suggested (Hasinoff, B.B., personal communication) that these curves might have a $kt^{1/2}$ dependence of the type found for high-viscosity experiments [16]. The residuals for the fit of this model, which gives a chi-square of 0.76, are shown in Fig. 4. The $\exp(-kt^{1/2})$ random-walk model proposed by Lindqvist et al. [6] fits with a chi-square of 0.26 and gives the residual plot shown in Fig. 5. Although the $t^{1/2}$ models do give improved fits compared with the single-exponential model, it is clear from the strongly systematic residual plots that neither is correct. However, when a double-exponential model is used an excellent fit is found with a chi-square of 0.11 and a completely random residual plot (Fig. 6). It must be emphasized that this better fit is not due to over-parameterization of the model. This is shown by the highest pair-wise correlation coefficient being only 0.778.

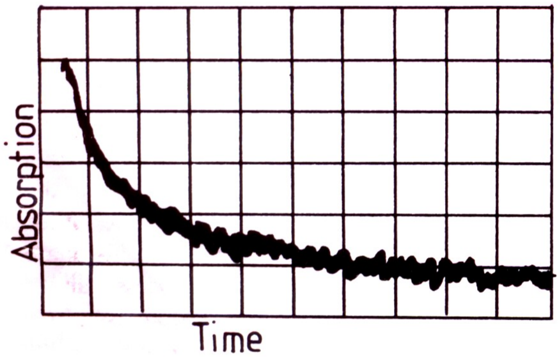

Fig. 2. 100 ns/division oscilloscope trace for photolysis of aqueous HbCO at 278 K with analysis wavelength 438 nm.

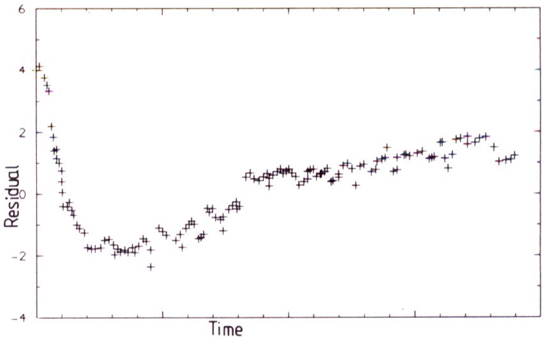

Fig. 3. Residual plot for analysis of Fig. 2 data as a single-exponential decay.

Now that an appropriate kinetic model has

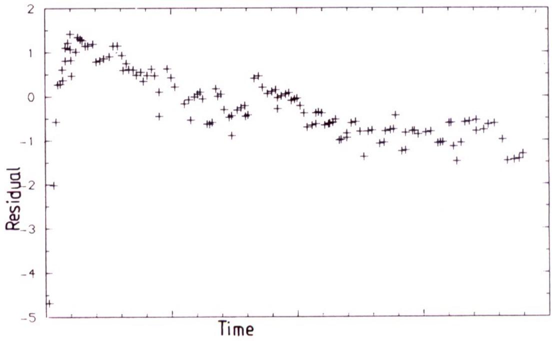

Fig. 4. Residual plot for analysis of Fig. 2 data to the model $1/\mathrm{conc.} = kt^{1/2} + 1/\mathrm{conc.}\ (t = 0)$.

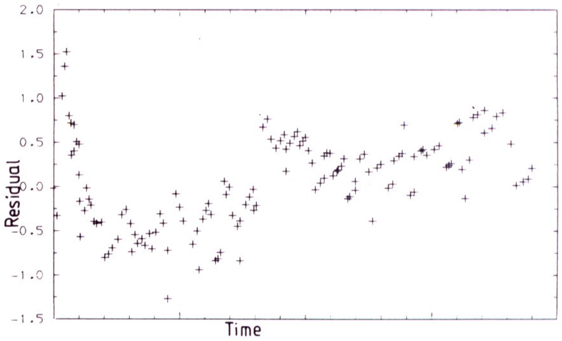

Fig. 5. Residual plot for analysis of Fig. 2 data to the model rate $\alpha \exp(-kt^{1/2})$.

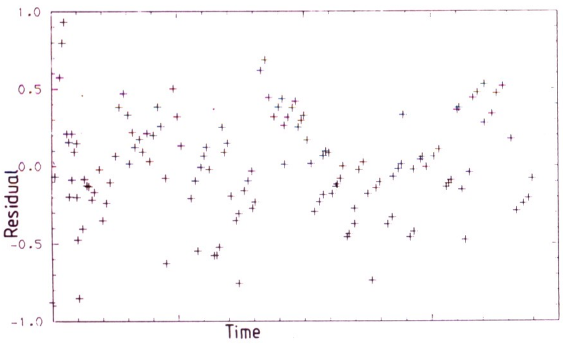

Fig. 6. Residual plot for analysis of Fig. 2 data as a double-exponential decay.

been established the parameters values obtained can be considered. At each temperature a number of traces have been analyzed: one set at 416 nm, where the absorbance increases with increasing ligand recombination, and one set at 438 nm, where absorbance decreases. The results from the double-exponential analysis are presented in Tables I and II. For each temperature, values of both the weighted mean and standard error of the mean are quoted for the rate constants and also for the pre-exponential ratios. The range of values obtained in each case is also given.

Although, as discussed above, these experiments were essentially free from certain sources of systematic error, two others remained. These arose because it was not possible to obtain a trace which at the same time allowed measurement of all experimental parameters and was suitable for kinetic analysis. Instead, values of I_0 and of the final-level absorption were measured separately from the coordinates. Errors in either of these would lead to systematic errors in the estimated rate constants.

The possible magnitudes of such errors were investigated by running a series of analyses on the same set of data with different constant values of I_0 or final level. The results showed that the values estimated for the rate-constant parameters were little affected by the constant value supplied for I_0. For example, a change of 20 mV, i.e., 10%, in I_0 led to only a 1% change in the rate constant. However, the value supplied for the final-level parameters was much more critical. A change of 1.5 mm in the final-level coordinate led to a change of approx. 7% in k_1 and approx. 25% in k_2. This dependence was also illustrated when the final-level parameter was treated as an adjustable parameter in the fitting equation. The results of the analysis showed high pair-wise correlation coefficients, in particular between the final-level parameter and k_2.

Because the error in the final level parameter used is likely to have been at least ±1 mm, it is clear that the quoted values of standard deviations for the best-fit parameter estimates are not meaningful quantities. This is evident in many cases where the range of values obtained exceeds

TABLE I

RATE PARAMETERS FOR ANALYSES AT $\lambda = 416$ nm

Temperature	k_1 (s^{-1})	k_2 (s^{-1})	Pre-exponential ratio
Mean	2.32E7 ± 0.109E7	0.752E6 ± 1.89E6	17.5 ± 7.89
308 K			
Range	2.11E7 − 2.81E7	−3.78E6 − 1.02E6	8.95 − 31.0
Mean	1.37E7 ± 0.0765E7	4.99E6 ± 0.254E6	1.61 ± 0.140
298 K			
Range	1.22E7 − 3.00E7	1.29E6 − 7.01E6	0.623 − 4.95
Mean	1.78E7 ± 0.0692E7	2.71E6 ± 0.154E6	1.76 ± 0.0947
288 K			
Range	1.40E7 − 2.23E7	2.49E6 − 3.11E6	1.16 − 1.94
Mean	1.38E7 ± 0.0561E7	2.58E6 ± 0.119E6	1.37 ± 0.312
278 K			
Range	1.21E7 − 1.84E7	1.51E6 − 3.06E6	0.994 − 2.82

TABLE II

RATE PARAMETERS FOR ANALYSES AT $\lambda = 438$ nm

Temperature	k_1 (s^{-1})	k_2 (s^{-1})	Pre-exponential ratio
Mean	3.96E7 ± 0.743E7	8.25E6 ± 1.65E6	1.64 ± 0.503
308 K			
Range	3.46E7–5.26E7	5.51E6–9.12E6	1.19–2.39
Mean	2.12E7 ± 0.162E7	4.58E6 ± 0.734E6	2.83 ± 0.539
298 K			
Range	1.90E7–2.52E7	2.53E6–5.75E6	1.92–5.06
Mean	1.85E7 ± 0.0640E7	3.20E6 ± 0.105E6	1.96 ± 0.829
288 K			
Range	1.47E7–2.17E7	1.72E6–4.54E6	1.58–4.55
Mean	1.65 ± 0.0375E7	1.85E6 ± 0.0375E6	1.62 ± 0.0331
278 K			
Range	1.56E7–1.73E7	1.64E6–2.23E6	1.48–1.75

that which would be consistent with the quoted errors. However, there is no reason to suppose that the values used for the final-level parameter were not subject to random error. Thus, it was meaningful to average the results of repeated experiments.

The errors in rate-constant values due to errors in the final-level parameter values would also account for the apparent discrepancies evident in certain cases between the mean rate-constant values for the sets of measurements at the two different wavelengths. Thus, the results cannot be taken to suggest that the processes observed at 416 nm differ from those observed at 438 nm. In consequence, the data have been combined for fitting to the Arrhenius equation to produce activation parameters.

Discussion

It would be desirable to be able to compare these results with those from the low-temperature studies of the individual single-chain proteins [9]. Unfortunately, it is not possible to compare critically the values for the rate constants obtained. The values obtained in this work are subject to considerable uncertainty, but they are extremely well-defined in comparison with the values calculated by extrapolation from the low-temperature studies. This uncertainty in extrapolation arises from the large errors in the Arrhenius frequency factors calculated from the rates measured at low temperatures. The converse is also true. The large errors associated with the values of the Arrhenius frequency factors calculated from the present study preclude meaningful extrapolation of these results outside the temperature range actually studied.

It is possible, however, to compare the values obtained for the activation energies. The faster process measured in the present study, having rate constant k_1, is found to have an activation energy of 6 ± 3.9 kJ · mol^{-1}. In the low-temperature work the fastest recombination step was designated B → A and the activation energies for this were found to be 4.6 and 4.3 kJ · mol^{-1} for the α and β chains, respectively. This last potential energy barrier in ligand binding occurs in the haem pocket and is assumed to be present at all temperatures. The actual nature of the barrier was associated with the energy needed to bring the iron atom from its out-of-place configuration (deoxy or T state) to its position in the plane of the porphyrin ring system (carbonmonoxyhaemoglobin R state) [9]. It would be very tempting to assume that, in view of the agreement between the activation energy value for our faster rate and that of the B → A recombination (observed in the low-temperature studies), we are looking at the same process.

The change from the stable R state to the stable T state involves movement of the iron atom out of the haem plane [17]. The question as to when this movement takes place is naturally intimately connected with the study of ligand photodissociation and recombination. Some early Raman spectroscopy studies were claimed to show that iron movement took place within a few nanoseconds following photodissociation [18,19]. However, other workers were less sure of their conclusions [20]. More recently the results of a picosecond resonance Raman investigation have been interpreted differently. Using 30-ps pulses from a synchronously pumped mode-locked cavity dumped dye laser (576 nm) to both photodissociate and probe a jet stream of carbonmonoxyhaemoglobin, they conclude that the spectrum of the photoproduct is already developed within 30 ps following photolysis, and furthermore remains unchanged for at least 20 ns after the pulse [21,22]. The spectra indicate that the iron atom in the photoproduct is high-spin, but is much closer to the haem plane than in deoxyhaemoglobin. The possibility of the larger high-spin iron (II) atom remaining in the haem plane is strongly supported by the recent X-ray structural determination of a stable porphyrin complex containing high-spin iron (II) in a pseudo-octahedral ligand geometry [23]. We must therefore conclude that both activation energies that we measure are due to other potential barriers present in the haem pocket, and that absorption changes observed on longer timescales and attributed to the R to T structural transition [24,13] must surely arise from the movement of the iron atom out of the plane. In other words Hb* is a species which could have a structure virtually identical to that of HbCO except for minor perturbations of the porphyrin structures due to the larger size of the high-spin iron (II) atom.

If this interpretation is correct, it is clear that the recombination scheme proposed to rationalize the results of the low-temperature photolysis studies is ill-founded, in that it assumes that the haem geometry has relaxed to the stable deoxy geometry before recombination occurs. The possibility of a metastable structure with an in-plane iron (II) atom has also been ignored in a recent theoretical treatment of ligand recombination [25]. More generally, the existence of this structure would mean that any detailed recombination scheme devised to explain the processes taking place close to the haem following photodissociation would not necessarily be applicable to the naturally occurring thermal ligand combination process.

Since we do measure two distinct activation energies (the one for the slower process having a value of 31 ± 4.8 kJ · mol^{-1}, which is in line with the values obtained at low temperatures for a type C → B activation energy in the proteins so far studied), can we correlate them with recombination either to individual α and β chains or by different pathways within a single chain? For example, it is well known from X-ray studies that the haem pocket is bigger in the α chain [17]. This type of datum has previously been used to correlate entropy factor differences between α and β chains for the B → A potential energy barrier in low-temperature studies [9]. The constancy of the pre-exponential ratio in Tables I and II would support two parallel processes. On the other hand, recent theoretical trajectory calculations based on X-ray crystal structures have demonstrated that groups of atoms have to relax in order to let small ligands such as oxygen and carbon monoxide escape from a myoglobin haem pocket [26]. Two major pathways were found, one parallel to the haem group through the E helix, the other perpendicular to the heme and out through the B helix. Calculations of the energy barriers along the parallel pathway give approximate values for the outer one of 25 kJ · mol^{-1} and for the inner one of 55 kJ · mol^{-1}. Our data at present do not allow a decision as to which of the alternatives is correct (reaction with individual chains or reaction via two pathways to an individual chain), but perhaps data with mutant haemoglobins [27] or with mixed ligand systems (carbon monoxide and non-photolabile nitric oxide) will enable a distinction to be made.

Finally, we believe that this study indicates that there is potentially far more information to be gained from detailed investigations at temperatures at and near to ambient temperature than the proponents of the low-temperature approach would have us believe [9]. When such direct observations are possible the results will be more reliable in that they do not involve extrapolations using an Arrhenius equation with very uncertain frequency factor estimates.

References

1 Alpert, B., Banerjee, R. and Lindqvist, L. (1972) Biochem. Biophys. Res. Commun. 46, 913–918

2 Alpert, B., Banerjee, R. and Lindqvist, L. (1974) Proc. Natl. Acad. Sci. U.S.A. 71, 558–562
3 Duddell, D.A., Morris, R.J. and Richards, J.T. (1979) J. Chem. Soc. Chem. Commun. 75–76
4 Duddell, D.A., Morris, R.J. and Richards, J.T. (1980) Biochim. Biophys. Acta 621, 1–8
5 Alpert, B., El Mohsni, S., Lindqvist, L. and Tfibel, F. (1979) Chem. Phys. Lett. 64, 11–16
6 Lindqvist, L., El Mohsni, S., Tfibel, F., Alpert, B. and Andre, J.C. (1981) Chem. Phys. Lett. 79, 525–528
7 Friedman, J.M. and Lyons, K.B. (1980) Nature 284, 570–572
8 Austin, R.H., Beeson, K.W., Eisenstein, L., Frauenfelder, H. and Gunsalus, I.C. (1975) Biochemistry 14, 5355–5373
9 Alberding, N., Chan, S.S., Eisenstein, L., Frauenfelder, H., Good, D., Gunsalus, I.C., Nordlund, T.M., Perutz, M.F., Reynolds, A.H. and Sorensen, L.B. (1978) Biochemistry 17, 43–51
10 Beece, D., Eisenstein, H., Frauenfelder, H., Good, D., Marden, M.C., Reinish, L., Reynolds, A.H., Sorenson, L.B. and Yue, K.T. (1980) Biochemistrey 19, 5147–5157.
11 Dye, J.L. and Nicely, V.A. (1971) J. Chem. Educ. 48, 443–448
12 Duddell, D.A., (1980) Photochem. Photobiol. 31, 121–127
13 Lindqvist, L., El Mohsni, S., Tfibel, F. and Alpert, B. (1980) Nature 288, 729–730
14 Wentworth, W.E. (1965) J. Chem. Educ. 42, 96–103
15 Bevington, P.R. (1969) Data Reduction and Error Analysis for the Physical Sciences, McGraw-Hill, New York
16 Hasinoff, B.B. (1981) J. Phys. Chem. 85, 526–531
17 Perutz, M.F. (1979) Annu. Rev. Biochem. 48, 327–386
18 Dallinger, R.F., Nestor, J.R. and Spiro, T.G. (1978) J. Am. Chem. Soc. 100, 6251–6252
19 Woodruff, W.H. and Farquharson, S. (1978) Science 201, 831–833
20 Lyons, K.B., Friedman, J.M. and Fleury, P.A. (1978) Nature 275, 565–566
21 Terner, J. Spiro, T.G., Nagumo, M., Nicol. M.F. and El-Sayed, M.A. (1980) J. Am. Chem. Soc. 102, 3238–3239
22 Terner, J., Stong, J.D., Spiro, T.G., Nagumo, M., Nicol, M. and El-Sayed, M.A. (1981) Proc. Natl. Acad. Sci. U.S.A. 78, 1313–1317
23 Reed, C.A., Mashiko, T., Scheidt, W.R., Spartalian, K. and Lang, G. (1980) J. Am. Chem. Soc. 102, 2302–2306
24 Sawicki, C.A. and Gibson, Q.H. (1976) J. Biol. Chem. 251, 1533–1542
25 Jortner, J. and Ulstrup, J. (1979) J. Am. Chem. Soc. 101, 3744–3754
26 Case, D.A. and Karplus, M. (1979) J. Mol. Biol. 132, 343–368
27 Giacometti, M., Di Iorio, E.G., Antonini, E., Brunori, M. and Winterhalter, K.H. (1977) Eur. J. Biochem. 75, 267–273