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EPR STUDIES OF PHOTOLYSIS OF NITROSYL HAEMOGLOBIN AT LOW TEMPERATURES: EFFECTS OF QUATERNARY STRUCTURE

by

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Abstract - Photolysis of nitrosyl haemoglobin (HbNO) has been studied from 6.5K to 20K for different NO saturation conditions. The kinetic curves are fitted equally well by a biphasic exponential and a distribution of activation energies. The parameters are straighforwardly related to the quaternary structure of the protein. The biphasic model indicates that two geminate processes in the NO reassociation to Hb dominate at low temperatures independently of the protein conformation.

Keywords: Photolysis; Nitrosyl Haemoglobin; Quaternary Structure; Electron Paramagnetic Ressonance

INTRODUCTION

In the last fifteen years special interest has been shown in the dynamics of proteins aiming at clarifying its relation to their physiological function. Heme proteins have been investigated by photodissociation kinetics[1-7] and spectroscopic experiments[8-11]. There are evidences for many possible intermediate states in the recombination of the ligand carbon monoxide (CO) to myoglobin (Mb)[5,11]. Kinetic studies are appropriate for identifying the time and temperature domains in which intermediate states can be isolated.

Low temperature experiments[1-3,5] and μs, ns and ps flash photolysis studies at high temperatures[4,6-8,10-11] have been carried out in order to establish the recombination kinetics and to characterize the geminate state. The mechanism of the ligand recombination has been usually determined from experiments involving a short laser pulse after which reassociation is monitored optically. The majority of these studies has been performed with CO, and sometimes O_2, bonded to the simplest haemoprotein Mb. The kinetics of photodissociated carboxy haemoglobin (HbCO) has been mostly studied at high temperatures because at such temperatures it is possible to observe structural changes from R to the T form[6-8]. It is interesting to observe the kinetics of the liganded Hb in the T structure. However this

form is stable only in special cases such as carp-Hb with CO and human-Hb with NO.

On the other hand NO has been used to test models for the kinetics and structure of HbO_2[4,15-17]. The outstanding aspect of NO is the possibility of using the Electron Paramagnetic Resonance (EPR) not applicable to other ligands. Its sensitive EPR spectrum makes it a convenient probe of the haem site environment. The EPR can be used as a complementary method for the investigations with NO[18-19]. In addition NO allows to study Hb in different quaternary conformations. It was shown that HbNO at low NO concentration is locked in the low affinity T state, while when saturated with NO it is in R state[13-14]. Intermediate conformational states can also be obtained. The change between R and T structures is easily identified by the characteristics of the EPR spectra[13]. EPR recombination studies of HbNO in the temperature range from 6.5K to 15.5K under continuous illumination have been fruitful[20], despite its low quantum yield. The basic concept of a distribution of activation energies in the tunneling regime[2] was successfully used.

Some optical experiments at room temperature were performed to explore the influence of different amino acids, and to study the similarities and differences between the fast geminate reaction in the tetrameric Hb and monomeric Mb

using NO as a ligand[4]. Detailed knowledge of protein motion at different time scales and temperature ranges is important to understand their reaction at the molecular level. Frauenfelder and co-workers[21] have shown that low temperature kinetic studies are relevant to determine the features of the geminate recombination.

As an extension of a previous paper[20] we report in the present work the kinetics of recombination of NO to Hb at different NO saturations. The EPR spectra of samples with R and T quaternary structures were studied under continuous illumination at low temperatures.

Experimental curves were fitted using a conformational substates model[1] and byphasic exponentials[3] to obtain activation energies and reassociation rates.

MATERIAL AND METHODS

Hb was prepared from fresh human blood using standard procedures. The Hb solutions were 0.4mM of heam in phosphate buffer 0.1M, pH 6.2. The HbNO in saturating condition (R conformations) was prepared as previously described[20]. We have used higher concentration and larger sample tubes than in our previous work[20] in order to improve the signal to noise ratio. HbNO in the T and mixed RT states was prepared in the presence of inositol hexaphosphate (IHP),

3 per haem, using two tonometers. One of them contained saturated HbNO and the other one a deoxygenated Hb solution. By means of a syringe under a nitrogen atmosphere, an appropriate small volume of HbNO is mixed with the deoxygenated one. The concentration of NO was calculated from the volume and concentration relations. This results to be about 7% of NO for the T structure and about 14% in the RT mixed conformation. After at least one hour, time enough for redistribution of NO among the haems, the samples were transferred to the EPR tubes and frozen.

A helium flux cryostat (Helitran LTD-3-110) with an APD-E temperature controller (both from Air Products and Chemical) were used to control the sample temperature. Temperatures were measured with an Au-Fe versus Chromel thermocouple placed on the sample tube wall. Temperatures were stable to within 1K.

EPR experiments were performed with a X-band spectrometer (Varian E-9). Spectra were obtained at low microwave power of 1.5×10^{-2} mW for R state and 4.9×10^{-2} mW for RT and T conformations with 2.0G modulation amplitude.

Photodissociation experiments were performed illuminating the sample through the window of the EPR cavity, with a 1000W Xenon Arc Lamp (Oriel Corp. of America, Stanford, CA) collimated and filtered through a saturated

copper sulphate solution. The light was left on for a time long enough for the EPR signal to reach a steady state.

The spectra were taken before illumination and in the steady state with the light on. Before repeating the experiment at a different temperature, the sample was warmed to about 77K so that the EPR signal intensity was recovered.

The kinetic curves were obtained from the registered changes of the EPR intensities at fixed g-values of the spectra as indicated by arrows in Fig.1. The curves were digitalized with a scanner (Digigraf) and normalized as in ref. 20.

The N(t) curves (normalized fraction of dissociated NO under illumination) were fitted with a sum of two exponentials given by equation

$$N(t) = 1-\left(\frac{A}{F_\infty}\exp\left(-(k_\nu+k_1)t \right) + \frac{B}{F_\infty}\exp\left(-(k_\nu+k_2)t \right)\right) \quad (1)$$

where $F_\infty = A + B$ is the steady state value of the NO dissociation fraction and A and B, the fractional amplitudes, $k_{1,2}$, the reassociation rates and k_ν, the dissociation constant. k_ν depends on the light intensity and sample concentration.

The fit of the experimental data of N(t) of the R state resulted in a constant value of A/F_∞ for all

temperatures. This led us to fix this fraction, with each corresponding F_∞, for the T and RT states, in order to adjust the k_1+k_ν and k_2+k_ν values.

The curves can also be fitted using a distribution of activation energies g(E) and the modified expression (equation (2)) for photolysis under illumination in the temperature range of tunneling regime[20]. The fraction $k_\nu/(k_\nu+k_L(E))$, where $k_L(E)$ is the reassociation rate under illumination, is the correction for partial photolysis.

$$N(t) = \frac{\int_0^\infty dEg(E)\ \frac{k_\nu}{k_\nu + k_L(E)}\left\{1 - \exp\left[-\left(k_\nu + k_L(E)\right)t\right]\right\}}{\int_0^\infty dEg(E)\ \frac{k_\nu}{k_\nu + k_L(E)}} \qquad (2)$$

where

$$g(E) = g_0 \exp\ \alpha\left\{\left(E^p - E\right) - n \exp\ \frac{\alpha}{n}\left(E^p - E\right)\right\}$$

We have used this distribution proposed by Frauenfelder[1], largely employed in the literature, because it allows for a direct comparison with several works. It was observed that other similar distributions do not yield better results[22-23].

The three parameters of the distribution, E^p (peak energy of the distribution), α, and n (parameters without

physical meaning), together with the reassociation rate k_ν, were adjusted to obtain the best fit to the kinetic curves for the entire set of temperatures. These fittings are good only if the barrier width d_0 and the frequency factor A are temperature dependent parameters. The fits were done by progressively decreasing the number of fitting parameters. At each step the parameter to be fixed was that with lowest error. The parameter is fixed equal to its mean value. The errors are given in Tables 2-4.

We have used a Monte Carlo like method (random numbers) to fit our data to equations (1) and (2) minimizing the root mean square error[20].

RESULTS AND DISCUSSION

The HbNO EPR spectrum is complex. Temperature and microwave power dependence studies have shown that for R state it is composed of at least three components[24]. One of these components dominates at very low microwave power and low temperatures. The EPR spectra before illumination and in the steady state are shown in Figure 1 for R, T and RT conformations. The experimental conditions are such that the EPR spectra are associated to only one component and their shape does not change with illumination. We failed to find an EPR spectrum of the photodissociated NO. Neither the

photoproduct nor differences in spectra during illumination were observed by EPR below 20K.

After turning off the light the fraction of NO which rebinds is very small for temperatures below 20K. We have analysed only the kinetic curves under illumination, that was shown to be sufficient to characterize the reaction[20].

Figure 2 shows curves of the dissociated fraction, F(t), during illumination for Hb in the R state at different temperatures. It is observed that the steady state value of F(t), F_∞, decreases with increasing temperatures. During prolonged photolysis, the species with high reassociation rates recombine, thus in the steady state the photolysed population is preferentially of the slow type.

This behavior is the same for the two other protein conformations. To see more clearly the differences between the three conformations we have plotted in Figure 3 their kinetic curves at similar temperatures. The T state reaches the steady state faster than R with a higher F_∞ value. It can be easily seen that going from T to R state, the steady state fraction of unbound NO is lowered as ligand affinity increases. This observed decrease is expected if low temperature kinetics can be related to ligand affinity in view of the room temperature results.

Dissociation in T state is observed at temperatures

higher than in RT conformation which in turn is observed at temperatures higher than R state. This result indicates that the barrier to NO decreases and/or is narrowed going from T to R conformations. The Figure 4a shows some fittings obtained with two exponentials using the parameters given in Table 1. We can see that the two rates differ about 10^2-10^3 fold. We assume that for each conformation the normalized fractions of photolysed molecules, A/F_∞ and B/F_∞, that rebind with slow (k_1) and fast (k_2) reaction rates, respectively, are temperature independent. The photolysed molecules are almost equally distributed in these two groups (fast and slow) as seen from the A/F_∞ values: 0.55 fo R, 0.4 for RT and 0.5 for T. As k_ν is temperature independent the weak temperature dependence of k_1 and k_2 (Table 1), when compared with the Arrhenius mechanism, suggests that quantum mechanical tunneling dominates, as expected, in the temperature range of the experiments. There is an evidence that below about 10K the reaction rates are constant probably associated to a single phonon mechanism.

The normalized kinetic curves treated with the conformational substates model in the tunneling regime, using equation (2) are shown in Figure 4b with parameters of the Tables 2-4. Figure 5 shows the activation energy distribution for R, RT and T conformations derived from these values.

The parameters yield k(E) values that are almost temperature independent below about 10K. This fact supports the hypothesis that the barrier tunneling is dominant in ligand recombination in this temperature range. The temperature dependence below 20K arises mainly from the pre-exponential A factor and is not appropriately described by an Arrhenius relation.

The k_ν values, obtained from the conformational substates model (Tables 2-4), are very low; they are consistently lower than the k_2+k_ν values obtained with the exponential model (Table 1). The high concentration and the absorption and scattering characteristic of the frozen sample contribute to the low values observed for k_ν. k_ν is sensitive to geometrical factors and to the copper filter concentration.

The characteristic barrier width d_0 decreases slightly with increasing temperatures. Although the largest change is no more than 30% in the RT conformation, the curves can not be fitted with a mean value of this parameter. The curves are very sensitive to d_0 changes. To explain this result we suppose that higher temperatures favour transitions to the excited states where the barrier width is smaller than in the ground state. Excited states with very short relaxation times (ps) were observed for MbCO[25,26] at room

temperatures. It was also observed that the protein does not relax or relaxes only partially to the unliganded form[27].

The parameters that characterize the parabolic barrier in the reassociation process are related in a straighforward way to the protein quaternary structure. That is, d_0 and E^p increase, going from R to T form, in agreement with the higher affinity of the R structure. This result is in accordance with the observed NO dissociated fraction at steady state, F_∞ (Table 1).

Cobau et al.[28] determined the E^p value for carp Hb in T conformation studying the reassociation of CO at 100K, 140K and 180K. They obtained 6.3kJ/mol and 7.5kJ/mol for R and T conformations, respectively. The relative difference between these values is much smaller than that found in the rebinding of NO to human Hb (Tables 2-4). This result indicates that the reaction of NO with iron is more sensitive to the structural changes than the CO reaction. It is possible that the difference between carp and human proteins also contributes to that difference.

It could be expected that the distribution g(E) for RT be a broader function resulting of the superposition of the individual R and T distribution. This is not observed in Figure 5. The tentative to combine the N(t) values of R and T to obtain the RT results was not successful either. This

discards the possibility of using appropriate combination of R and T distribution to fit the experimental kinetic curve of the RT conformation. This result indicates that Hb can take different intermediary RT conformations between the R and T states.

CONCLUSIONS

At low temperatures power law kinetics, obtained from a distribution of activation energies or from the sum of two exponentials are indistinguishable. The data can be fitted equally well by both models as shown in Figures 4 and 6. The correctness of fits are judged by the residuals, differences between experimental and calculated values, shown as examples in Figure 6. The analysis of all kinetic curves confirms that both models are actually indistinguishable.

The fact that two reassociation rates are sufficient to describe the kinetic curves indicates that two particular conformations predominate in the equilibrium distribution. Cornelius et al.[15], in their picosecond photolysis work, at room temperature, have also observed two geminate processes for NO ligand in Hb. The ratio between the two reassociation rates is about 6, close to that observed by Jongeward et al.[4] for sperm whale MbNO in the ps range. In this case it was impossible to mesure the geminate

recombination rate in the ns range. For other ligands the two processes that occur in completely different time ranges have rates differing by a factor of 10^2-10^3, similar to our results. The continuous illumination favours the slow component population and allows for the determination of its rate.

The necessity of invoking a minimun of two geminate conformers is not surprising as it has been reported that EPR spectra of saturated HbNO solution show at least three components that dominate at different temperatures and for different microwave power conditions[24]. We suggest that these components are responsible for different kinetic properties.

Although the present results give some insight into the problem, the complexity of HbNO system requires a more elaborate model and further experiments. The unusual properties of the EPR spectrum such as its strong dependence on microwave power give further importance to experiments on recombination as a function of this parameter.

We believe that further comparison studies can contribute to the understanding of the nature of geminate recombination. This work provides qualitative relationship among kinetic parameters and quaternary structure and evidences that low temperature recombination is dependent on the globin conformation.

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Figure captions

Figure 1 - EPR spectra of HbNO. Upper curve of each set, before illumination and lower curve, in the steady state. R conformation (Hb saturated with NO) at 7K and $1.5x10^{-2}$mW microwave power, RT (Hb with 14% of NO in the presence of IHP) at 10.1K and $4.9x10^{-2}$mW and T (Hb with 7% of NO in the presence of IHP) at 10.4K and $4.9x10^{-2}$mW; 2.0G modulation amplitude.

Figure 2 - Temperature dependence of NO dissociated fraction, F(t), under illumination, for Hb in the R conformation.

Figure 3 - NO dissociated fraction, F(t), under illumination, for the three conformations.

Figure 4 - Normalized kinetics curves of HbNO T conformation at 16.2K (0.4mM phosphate buffer 0.1M, pH=6.2, 3IHP:Fe). a) fitting with the two exponentials model (equation (1)), with parameters of Table 1; b) fitting with the conformational substates model (equation (2)), with parameters of Table 4.

Figure 5 - Activation energy distribution g(E) for the three conformations with parameters of Tables 2-4.

Figure 6 - Residuals of the fits of the exponential (dashed line) and distribution (solid line) models for: a) R state at 12.3K; b) RT mixed state at 15.5K; c) T state at 10.4K and d) T state at 16.2K

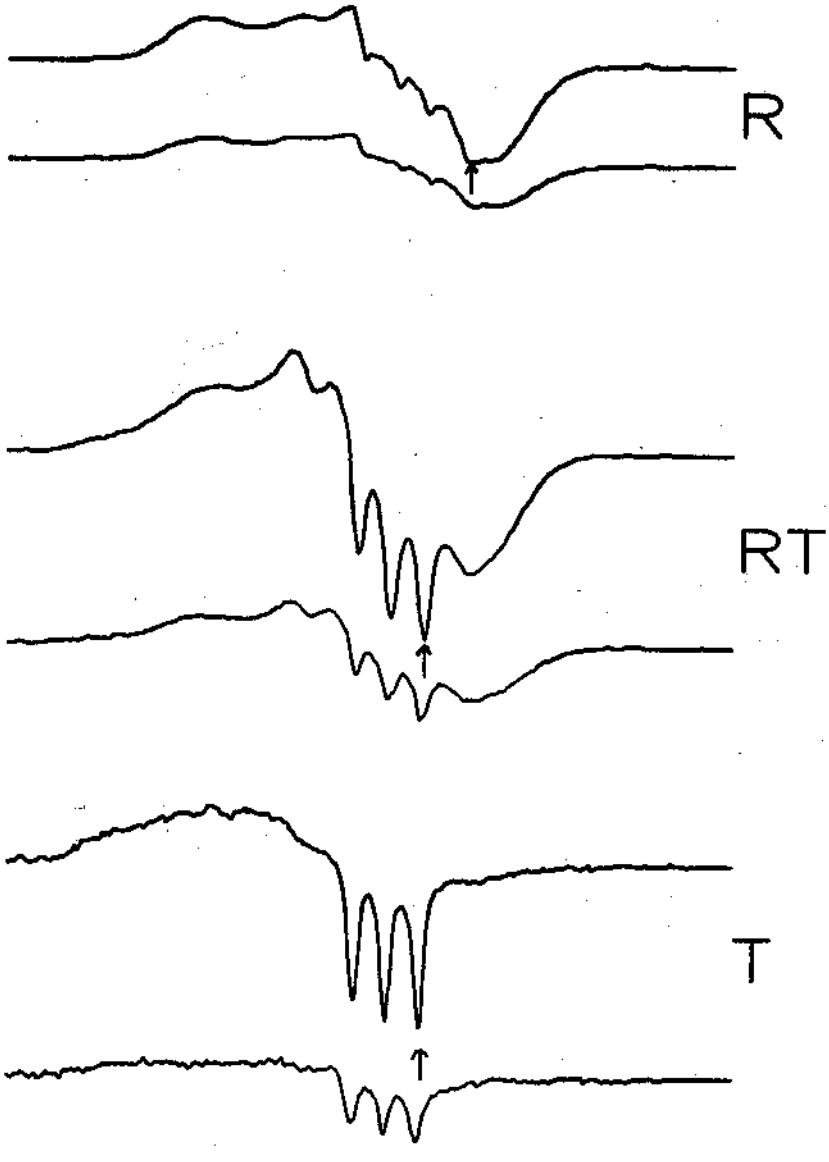

Figure 1

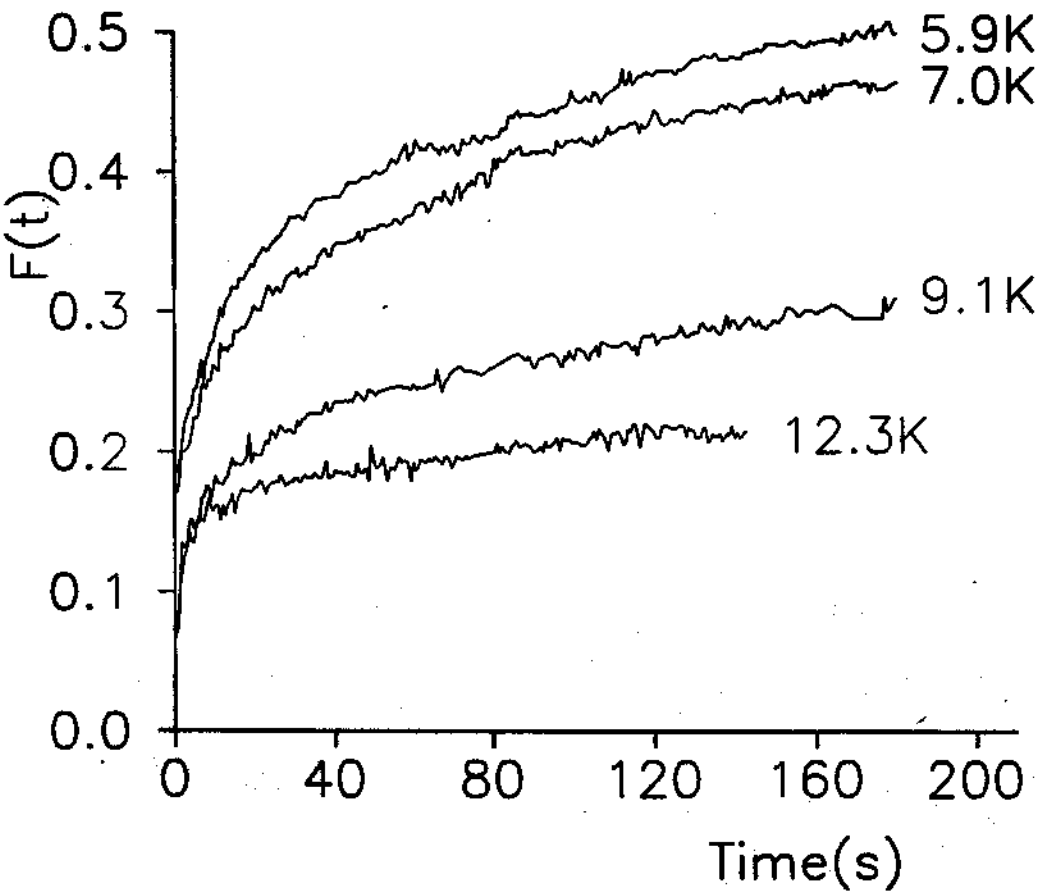

Figure 2

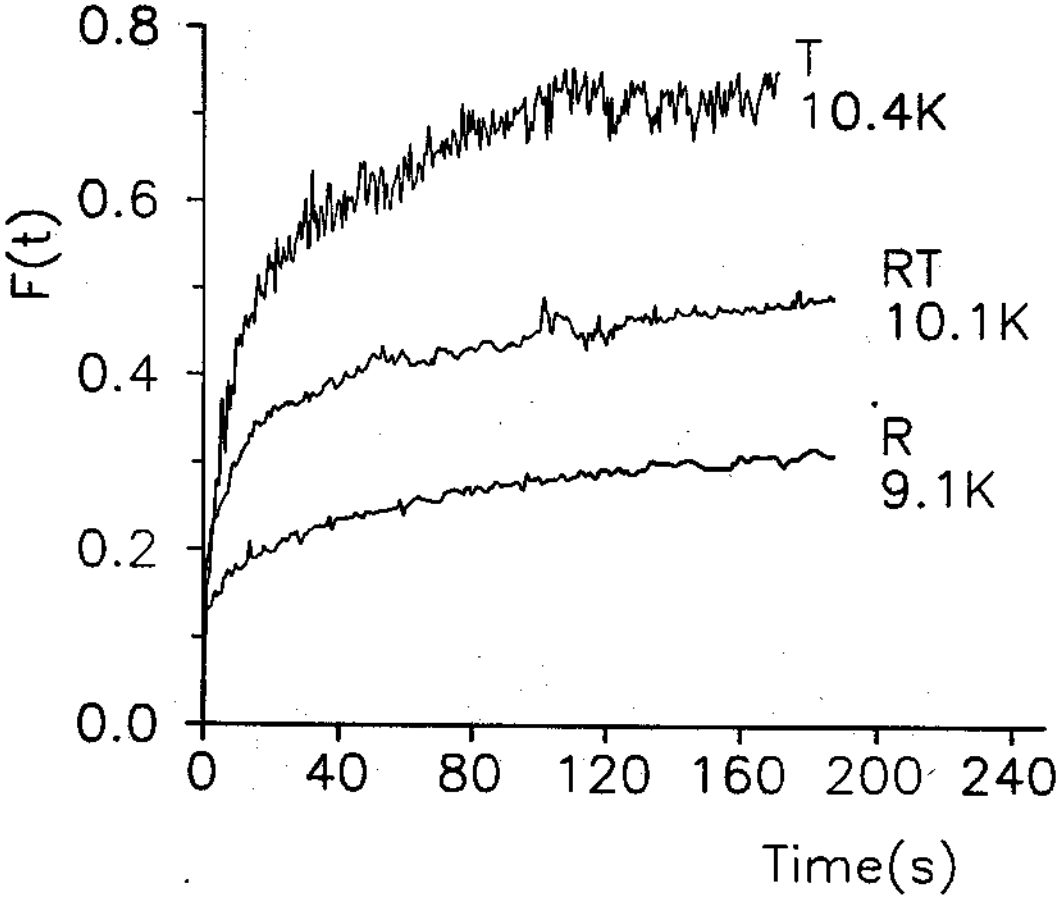

Figure 3

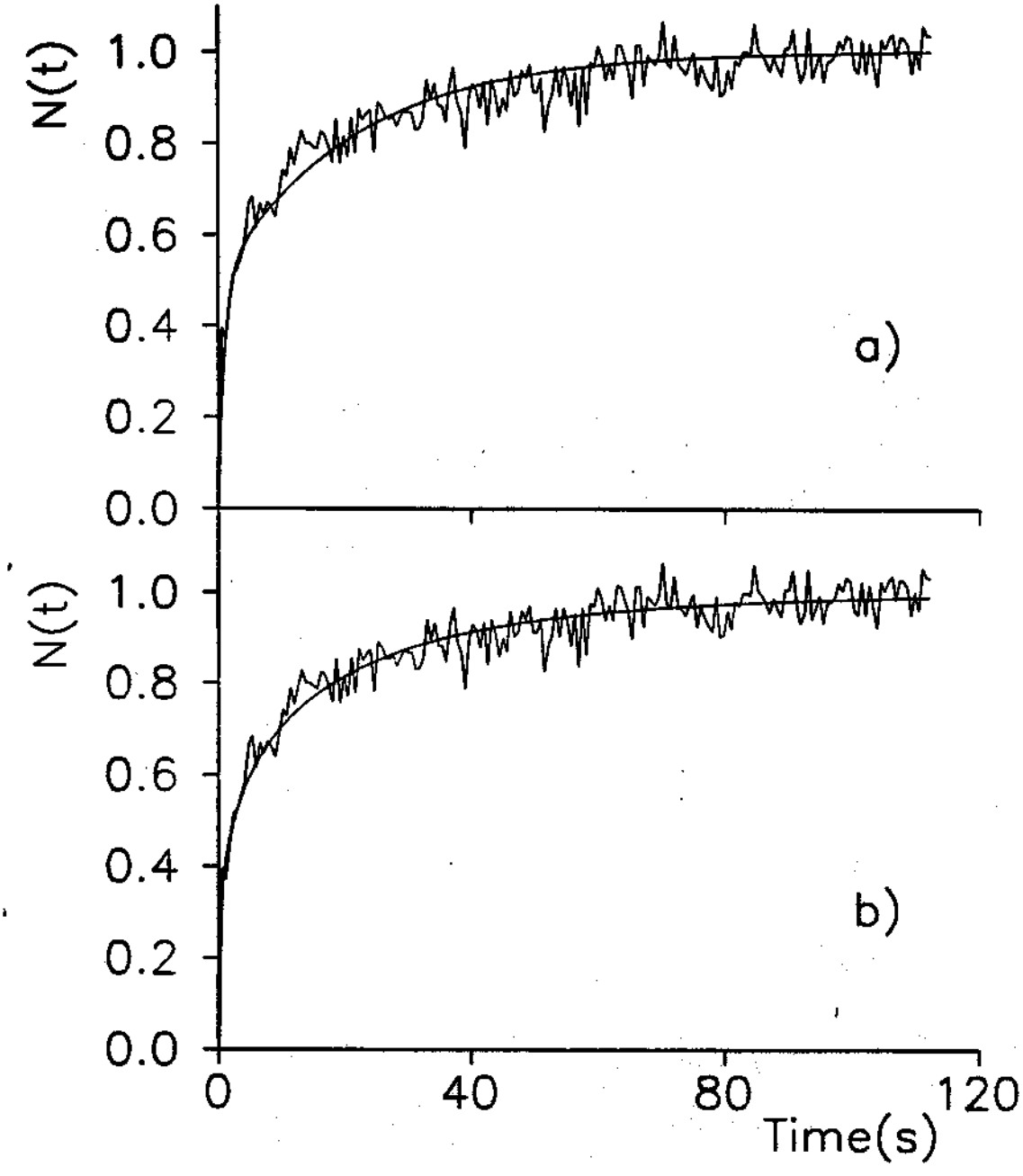

Figure 4

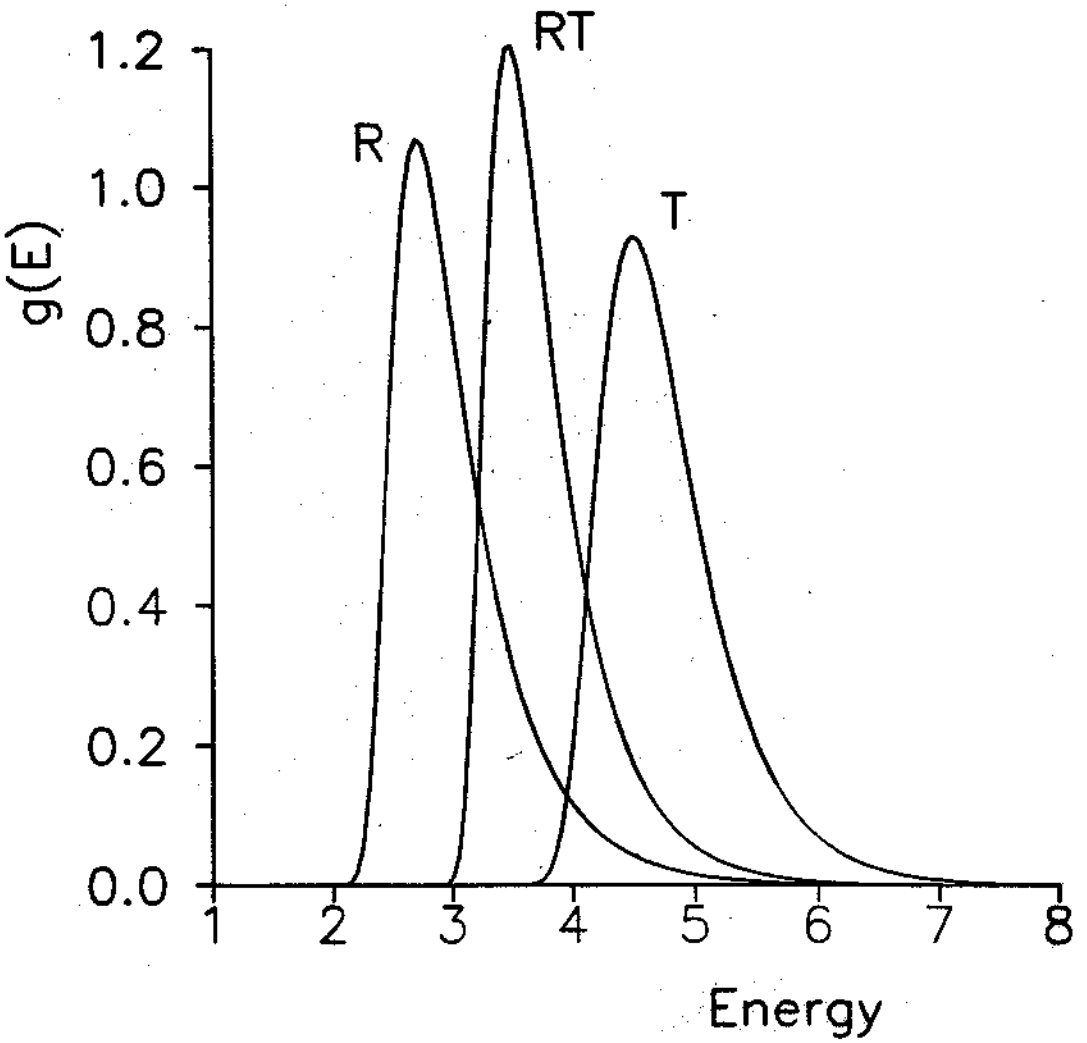

Figure 5

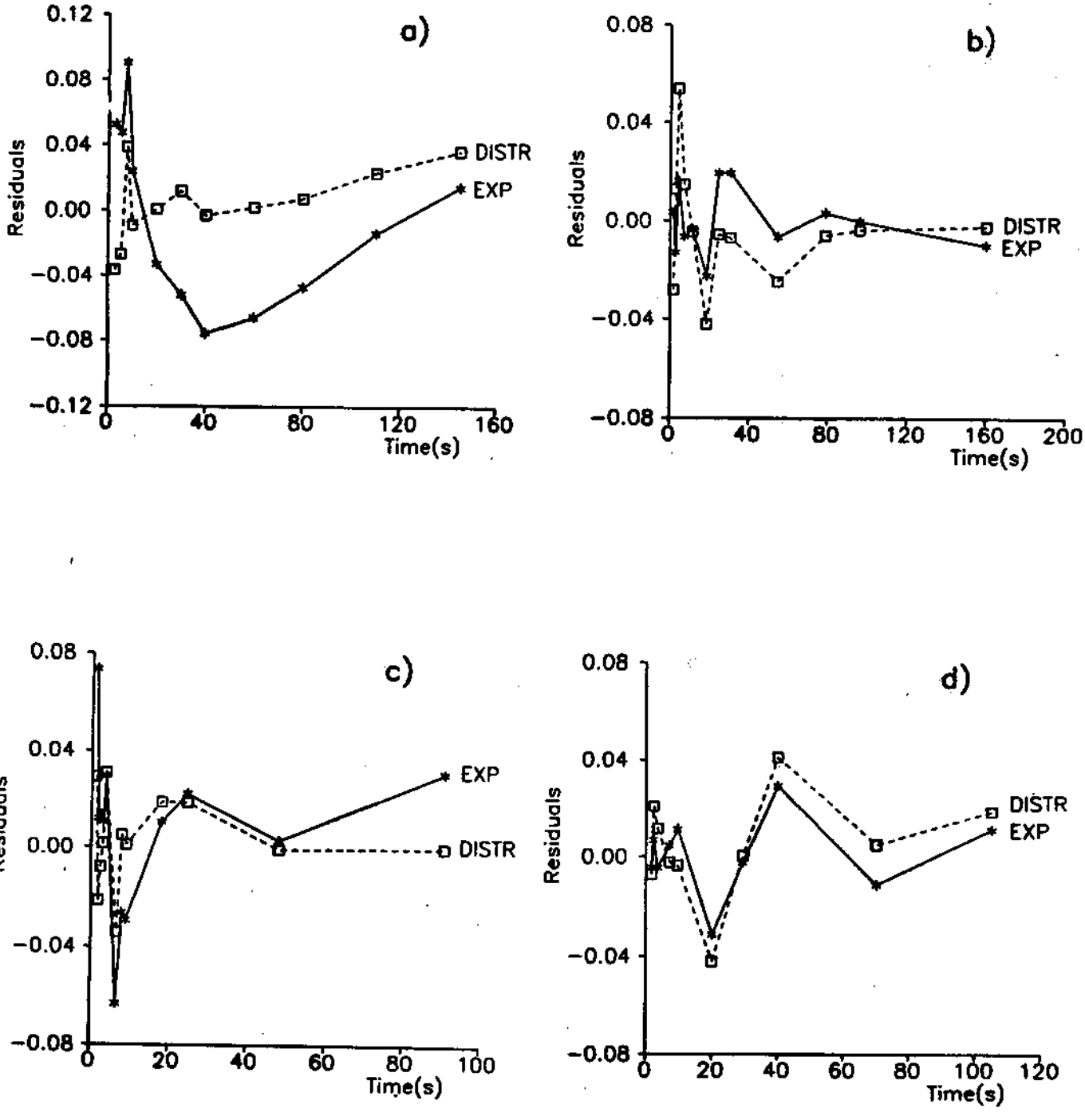
a)
b)
c)
d)
Residuals
Time(s)
DISTR
EXP
0.12
0.08
0.04
0.00
−0.04
−0.08
−0.12
0
40
80
120
160
200
20
60
100

Figure 6

Table 1 - Parameters obtained by fitting the kinetics curves with two exponentials model for the R, RT and T conformations of HbNO.

Temperature (K)	F_∞	$k_\nu+k_1$	$k_\nu+k_2$
R conformation - $A/F_\infty = 0.55 \pm 0.02$			
5.9	0.503	0.0209	0.961
7.0	0.466	0.0178	1.461
9.1	0.310	0.0191	1.218
12.3	0.211	0.0625	3.450
RT conformation - $A/F_\infty = 0.40 \pm 0.05$			
6.5	0.600	0.0087	0.270
10.1	0.500	0.0106	0.380
13.4	0.390	0.0194	0.887
15.5	0.310	0.0226	1.035
T conformation - $A/F_\infty = 0.50 \pm 0.1$			
10.4	0.685	0.0285	0.296
16.2	0.447	0.0467	0.985
17.4	0.394	0.0517	1.867
19.6	0.311	0.0871	1.880

Table 2 - R conformation of HbNO. The temperature dependence of the barrier width and the frequency factor obtained from kinetic curves[a].

Temperature (K)	d_0(nm)	log [A(s^{-1})]
5.9	0.0142	3.84
7.0	0.0145	3.87
9.1	0.0142	3.84
12.3	0.0111	4.29

[a]Fitting parameters using the distribution function g(E): E^p = 2.71 ± 0.02kJ/mol, α = 2 ± 0.3mol/kJ, n = 0.39 ± 0.03 and k_ν = 0.0098 ± 0.0002s^{-1}.

Table 3 - RT conformation of HbNO. The temperature dependence of the barrier width and the frequency factor dependence obtained from kinetic curves[a].

Temperature (K)	d_0(nm)	log [A(s^{-1})]
6.5	0.0197	5.75
10.1	0.0182	
13.4	0.0162	5.92
15.5	0.0157	

[a]Fitting parameters using the distribution function g(E): E^p = 3.5 ± 0.2kJ/mol, α = 2.30 ± 0.2mol/kJ, n = 0.40 ± 0.9 and k_ν = 0.0084 ± 0.0004s^{-1}.

Table 4 - T conformation of HbNO. The temperature dependence of the barrier width and the frequency factor obtained from kinetic curves[a].

Temperature (K)	d_0 (nm)	log [A(s^{-1})]
10.4	0.0247	3.84
16.2	0.0214	
17.4	0.0207	7.5
19.6	0.0204	

[a]Fitting parameters using the distribution function g(E): E^p = 4.50 ± 0.1kJ/mol, α = 2.20 ± 0.5mol/kJ, n = 0.7 ± 0.1 and k_ν = 0.0188 ± 0.003s^{-1}.

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