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Spin-lattice relaxation of denatured nitrosyl hemoproteins

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ABSTRACT

The temperature dependence of spin-lattice relaxation (SLR) for denatured nitrosyl hemoglobin (HbNO) and nitrosyl Myoglobin (MbNO), powdered HbNO and hematin-NO was studied in the range from 4K to 70K. The results were fitted with both T^n and $e^{-\Delta/T}$ models. In the first one the relaxation is mediated by tunneling modes of a two level system. We observed a correlation between the n values and the functional state of protein. The stricking coincidence of the range of the low lying energy level and the temperature range where EPR spectra changes suggest the existence of two conformation of the liganded heme. The relevance of the presence and structure of the globin is revealed itself in the difference between parameters determined for native, hematin and denatured proteins.

Key-words: Denatured hemoproteins; Cross relaxation; Conformational states.

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Introduction

The temperature dependence of the spin-lattice relaxation (SLR) time, T_1, of metallproteins have been characterized by an anomalous T^n temperature dependence for the Raman rate, with n related to the fractal dimension of the entire polimers.[1] Relaxation of denatured azide methemoglobin has given further suport to the effect of conformational changes on the relaxation process.[2] In particular, more recently, the iron in nitrosyl hemoproteins has been subject of interest in relaxation studies.[3–6]

It was first observed that nitrosyl myoglobin (MbNO) in solution at low temperatures (4.2K to 20K) presents a linear temperature dependence for the SLR rate, T_1^{-1}, without any attempt to identify the relaxation mechanism.[4] More recently, measurements over a wide temperature range have shown an anomalous T^n power law with $n = 2.2 \pm 0.3$ in solution and powdered samples.[5] This result was attributed to the tunneling within localized states[7,8] associated to the existence of conformational equilibrium in hemoproteins.

The power saturation between 1.4K and 4.2K and temperature dependence above 80 K of EPR spectra of nitrosyl hemoglobin (HbNO) crystals show a T_1 and spin-spin relaxation time, T_2, behaviour that was explained qualitatively by a relaxation process that involve heme-heme magnetic dipolar interactions between both kinds of subunits.[3] On the other hand, Electron Paramagnetic Resonance (EPR) spectra of HbNO solution were studied in the temperature range from 7.5K to 104K. They are composed of at least three components (A, B and C) which intensities have different dependence on temperature and microwave power level.[6] Their relaxation behaviour was studied by continous saturation method and the temperature dependence of T_1 for the A component (low temperature and low power level component) showed to follow an Orbach-like mechanism with a characteristic energy of $\Delta = 28$ cm^{-1}. It was proposed that this energy refers to a difference between two different geometries of liganded heme.

Nitrosylhemoproteins do not present a Raman relaxation mechanism with a fractal exponent, even so, it is still particularly interesting in giving information on two level systems. These results stimulated the investigation of the SLR mechanism of denatured hemeproteins, seeking for information on excitations and dynamics of their disrupted structure which has no physiological function.

This works aims to verify the effect of protein denaturation, as well as the importance of globin presence on the SLR mechanism. The present paper is a complementar study

on the temperature dependence of the SLR time of hemoproteins, by the observation of heat denatured MbNO and HbNO, powdered HbNO and nitrosyl hematin (hematin-NO).

Experimental

Hb was prepared from fresh human blood using standart procedures. Mb (horse heart, Sigma) was completely oxydized with $K_3Fe(CN)_6$ and the excess was removed by gel filtration. Hematin (Sigma) was reduced with excess (2:1) sodium dithionite and kept in anaerobic conditions with a N_2 flux. Solutions were 1 to 2.5 mM of heme in phosphate buffer 0.1 M, pH 7 except for hematin which was dissolved in pyridine. Nitrosylation was obtained by introducing nitric oxide (NO) gas directly into the previously deareted EPR sample tube. Heat denatured MbNO and HbNO were prepared at 80°C for 5 h.[9] Powdered samples was prepared using a Labconco 75200 lyophilizer.

EPR masurements were perfomed in a E-9 Varian X- band spectrometer from 4K to 100K using a helium flux cryostat (Helitran LTR-3-110). Temperatures were measured with a Au-Fe vs chromel thermocouple placed below the sample.

The half-saturation powers, $P_{\frac{1}{2}}$, and the SLR rate T_1^{-1} were estimated as previously described, using the continous saturation method.[10] The intensities of the $g = 2.07$ and $g = 2.011$ EPR lines in denatured samples and only $g = 2.07$ signal in hematin were monitored as indicated in the spectra of fig. 1.

The saturation conditions do not allow the determination of $P_{1/2}$ for temperatures higher than 70 K. Then the temperature dependence of T_1^{-1} was fitted to either one of the expressions bellow over the temperature range from 4K to 70K.

$$T_1^{-1}(\text{arbitrary units}) = AT^n + C \tag{1}$$

$$T_1^{-1}(\text{arbitrary units}) = B \exp\left(-\frac{\Delta}{T}\right) + D \tag{2}$$

where n, A and B, Δ and D are fitting parametres. The fitting procedure pointed out the necessity of the temperature-independent terms C or D.

Results and Discussion

The EPR spectra were obtained as a function of temperature and microwave power. In fig. 1, the EPR spectra of denatured MbNO and HbNO, and powdered HbNO at 6K and 20 dB ($\simeq$ 1.3 mW) are presented. The spectra are asymetric and show a well resolved three line hyperfine structure characteristic of the penta-coordinated iron.[11] We observed that the spectral shape does not depend neither on temperature nor on microwave power.

EPR spectra of hematin-NO as a function of temperature at 22 dB are shown in fig. 2. We noticed that for temperatures above 77K the spectrum undergoes a change in shape similar to that observed in native HbNO at lower tamperatures, about 30 K.[6] For temperatures lower than 77K, the spectra do not depend on the temperature, but they present a negligable dependence on microwave power (spectra not shown). Then only the high field line, $g = 2.07$, that has less contribution from other components was used for saturation measurements.

In fig. 3 the temperature dependences of the SLR rates are presented for denatured HbNO (fig 3a), denatured MbNO (fig. 3b), powdered HbNO (fig. 3c) and hematin-NO (fig. 3d). The solid and dashed lines correspond to the best fits of equations 1 and 2, respectively. It is seen that except for hematin-NO both fittings of the experimental data are equivalent within the experimental error. Analysis of the residual differences between experimental and calculated values confirms that both fits are actually indistinguishable unless for hematin-NO which is best fitted by the T^n function (fig. 4d).

For comparison, the parameters of these fits are listed in table 1, togheter with those from references.[5,6]

Except for HbNO solution table 1 evidences a T^n dependence for the SLR rate with n = 2.2 $\pm$ 0.2 for native and powdered samples. In the denatured samples and hematin this exponent increases to 3.8 $\pm$ 0.4. This temperature dependence is associated to a model based in a relaxation mediated by tunneling modes of a two level system. The TLS (tunneling localized state) proposed for powdered MbNO[5] can be extended to powdered HbNO, since frozen solutions of this complex also exhibits more than one binding conformation associated to the two-level system.[6,12]

The deviation of n from the value of 2, λ, gives in this model the relation between the density of states and the energy in the form $\rho(E) \approx E^{\lambda}$ and is related to the value of the characteristic energy E_{max}, the maximun energy of TLS or the cutoff in the distribution of

the asymetry parameter of the double well potencial. There is a correlation between the λ values obtained and the functional state of the protein. Native and powdered samples, which are reversible to native, presents low λ values (0.2 to 0.4) when compared to the values of denatured proteins, hematin or to the values of 1.5 to 1.6 of inorganic amorphous materials.[7]

If we consider the dependence given by equation 2 it provides evidence for a low lying energy level of 65 cm^{-1} for denatured HbNO, 51 and 77 cm^{-1} for denatured MbNO and of 75 cm^{-1} for hematin-NO. These values are higher than that obatined for the low power component of native HbNO, 28 cm^{-1},[6] but it is still much lower than the difference in energy between the ground and excited eletronic states in the proposed energy level diagram of nitrosyl hemoproteins.[13]

It is interesting to observe that native HbNO presents a low lying energy level of 28 cm^{-1} for A component and its EPR spectra changes about 32K,[6] B component (low temperature and high microwave power component) disappears and another one called C appears. Similarly our results indicate a low energy level of 74 cm^{-1} for hematin-NO with EPR spectra changes at temperatures about 80K.

Walleh et al have shown that the observed variations in EPR spectra for heme proteins from various sources and under different experimental conditions can be accounted for in terms of changes in the ligand geometry, which may induced by the protein.[14] Then the values above determined from SLR and temperature dependence of the EPR spectra can be related to a difference between two conformation (geometry) of the liganded heme, produced by different axial ligand distances and Fe-N-O bond angles. The energy values are in good agreement with the range of 10-100 cm^{-1} predicted and observed for low frequency phonon modes of the exterior medium for Mb by ineslatic neutron scatering.[15,16] Then the variation in the ligand geometry could be induced by these phonons.

Although denatured HbNO and MbNO and powdered HbNO do not present variations in the EPR spectra, the observed low lying energy state suggets the existence of a second conformational state not detectable by EPR. This is strengthned by the anomalous temperature dependence of the intensity of EPR spectra of these complex which was attribute to the equilibrium between two different conformations.[12]

The λ values obtained above for the TLS model correlate to the energy difference between the conformations in equilibrium. A higher cutoff of the asymetry parameter is

associated to a higher low lying energy state.

At this point we considered eq. 1 to evaluate T_1 absolute values. This choice considers that this equation fits almost all samples analyzed in table 1. Equation 2 would not yield significant different results. Absolute values of T_1(s) can be estimated considering the linewidth, and the Q factor of tha cavity.[17,18]

In table 2 data are presented for $T_1^{-1}(s^{-1})$ at three different temperatures, where the contribution of the two terms of equation 1 were calculated separately.

These results supported by Fig. 3 show that at temperatures lower than 11 ± 3K the main contribution to the relaxation of denatured and hematin samples is due to the constant term. This term was not observed neither in native nor in powdered NO hemoproteins.[5] This behaviour could be explained considering that in the broken structure of denatured samples and in hematin the interheme distances are sufficiently short to the spin-lattice relaxation be dominated by cross relaxation between hemes at low temperatures.

Cross relaxation is possible only if the transitions have overlapping lines.[19] The cross relaxation rate T_{1c}^{-1} modulated by dipole interaction can be calculated using the values obtained by Bloembergen et al for the overlap of Gaussian resonance lines[20,21]

$$T_{1c}^{-1} = \frac{g^3\mu_B^3}{2\pi^{1/2}\hbar H r^6}[S(S+1)(1-\cos^2\theta)]^2 \qquad (3)$$

where μ_B is the Bohr's magneton, $\hbar$ is the Planck's constant, r is the mean distance between heme groups, H is the magnetic field and θ is the angle that the radius vector between two neighbouring sites makes with the magnetic field.

Using the constant term in table 2 in equation 3 we estimate the distance between heme groups. For g=2.07 it results 21 Å for hematin-NO and 12 Å for denatured HbNO and MbNO.

The distance between the iron atoms in the R structure of HbNO is $Fe(\alpha_1 - \alpha_2) = 36$ Å and $Fe(\beta_1 - \beta_2) = 33$ Å, while the T structure the corresponding distances are 35 Å and 40 Å.[22] A relative decrease of the distance between heme groups in denatured and hematin-NO samples as compared to the values in R or T structure of HbNO, together with the total overlap between the spectral functions make cross relaxation possible in those samples.

We can roughly estimate from volume changes and cristalographic data the changes in the distance between hemes due to the denaturation and dehydration processes. If

	g	A	n	C	B	Δ (cm $^{-1}$)	D	Ref[a].
native HbNO	—	—	—	—	$10^{5.1}$	28	—	6
powdered HbNO	2.064	0.11	1.9	—	928	51	4.2	This work
	2.003	0.048	2.2	—	1120	47	2.3	This work
denatured HbNO	2.07	0.0003	3.6	3.9	1566	65	4.4	This work
	2.011	0.0002	3.6	2.0	785	65	2.3	This work
Native MbNO	2.007	—	2.4	—	—	—	—	5
powdered MbNO	2.064	—	2.4	—	—	—	—	5
	2.003	—	2.1	—	—	—	—	5
denatured MbNO	2.07	0.003	3.5	3.8	618	51	4.4	This work
	2.011	0.00001	4.5	1.3	1836	77	1.0	This work
hematin-NO	2.07	0.00002	3.7	0.1	325	75	0.1	This work

Table 1: Fitting parameters of T_1^{-1} according to equations 1 and 2. [a] In references 5 and 6 only the best fitting was considered and the constant term was not observed.

	$AT^n \times 10^6$	$C \times 10^6$	$T_1^{-1} \times 10^6$
Hematin-NO — g = 2.07			
5K	0.1	0.7	0.8
10K	0.7	0.7	1.4
15K	3.1	0.7	3.8
denatured MbNO — g = 2.07			
5K	5.9	25	30.9
10K	69	25	94
15K	288	25	313
denatured HbNO — g = 2.07			
5K	0.6	24	24.6
10K	7.1	24	31.1
15K	30	24	54

Table 2: Absolute values of T_1^{-1} (s^{-1}) according to equation 1.

we consider no changes in the relative positions of the heme and in the volume of the proteins, the mean distance between heme in powdered samples is equal or smaller than in denatured ones, however the constant term related to cross relaxation is not observed in powdered samples. The results above is in agreement with a drastic change in the protein structure during danaturation process, leaving the heme groups closer in denatured than in powdered samples.

It has been pointed out that the effect of dehydration of MbNO is to increase the SLR rate from 5.6 $\times 10^3 s^{-1}$ in solution to 1.8 $\times 10^4 s^{-1}$ in powdered MbNO at 20 K.[5] This effect is strengthened in HbNO shown by the increase of the SLR rate from 1.6 $\times 10^4 s^{-1}$ in solution to 1.8 $\times 10^8 s^{-1}$ at least in powdered HbNO. We observed that the SLR rate increases further in denatured MbNO relative to powdered MbNO. This effect is also observed in the equivalent HbNO samples. The values of T_1^{-1} at 20 K are 1.1 $\times 10^8 s^{-1}$ and 7.9 $\times 10^8 s^{-1}$ for denatured MbNO and HbNO, respectively. It is related with the fact that the denaturation process can go further than dehydration, as previously observed in metHb. In both processes we can observe the formation of hemichrome P, but in denaturation there is one more structural change distorting hemichrome P into P', with different EPR spectra.[9]

Both models seen able to explain our results. The exponential one gives informations about the influence of neighbours on the SLR mechanism and suggests a relation between low lying energy level and changes of conformation in liganded heme. On the other hand in the tunneling localized states model the importance of structure-function relationship is observed and the similarity between proteins and glasses is strenghened. In the T^n temperature dependence for the Raman rate in low-spin Fe(III) proteins, the n value was firstly interpreted in terms of the fractal dimensions of the proteins.[1] More recently studies of cooper as well as iron proteins shown that the relaxation behavior is not correlated with the chain fractal dimensions of protein.[23] The similarity in the n value of Raman rate for low spin Fe(III) in heme proteins and small molecule low spin Fe(III) porphyrin suggests that n is determined more by the local heme environment than by the long range structure of protein.[24] Nevertheless, our results show that relaxation processes in nitrosil hemoproteins, where the n value of TLS model and cross relaxation can be observed or not, are related to the presence and structure of globin.

Figure Captions

Fig. 1: EPR Spectra of different samples at 6K and 20 dB; (a) denatured MbNO, (b) denatured HbNO and (c) powdered HbNO.

Fig. 2: EPR spectra of hematin-NO as a function of temperatures at 22 dB.

Fig. 3: The temperature dependence of the SLR rates of g=2.07 for (a) denatured HbNO, (b) denatured MbNO, (c) powdered HbNO and (d) hematin-NO. - - - - T^n and ——- $e^{-\Delta/T}$ models.

Fig.4: Residuals differences between experimental and - - - - T^n and —— $e^{-\Delta/T}$ models for SLR rates; (a) denatured HbNO, (b) denatured MbNO, (c) powdered HbNO and (d) hematin-NO.

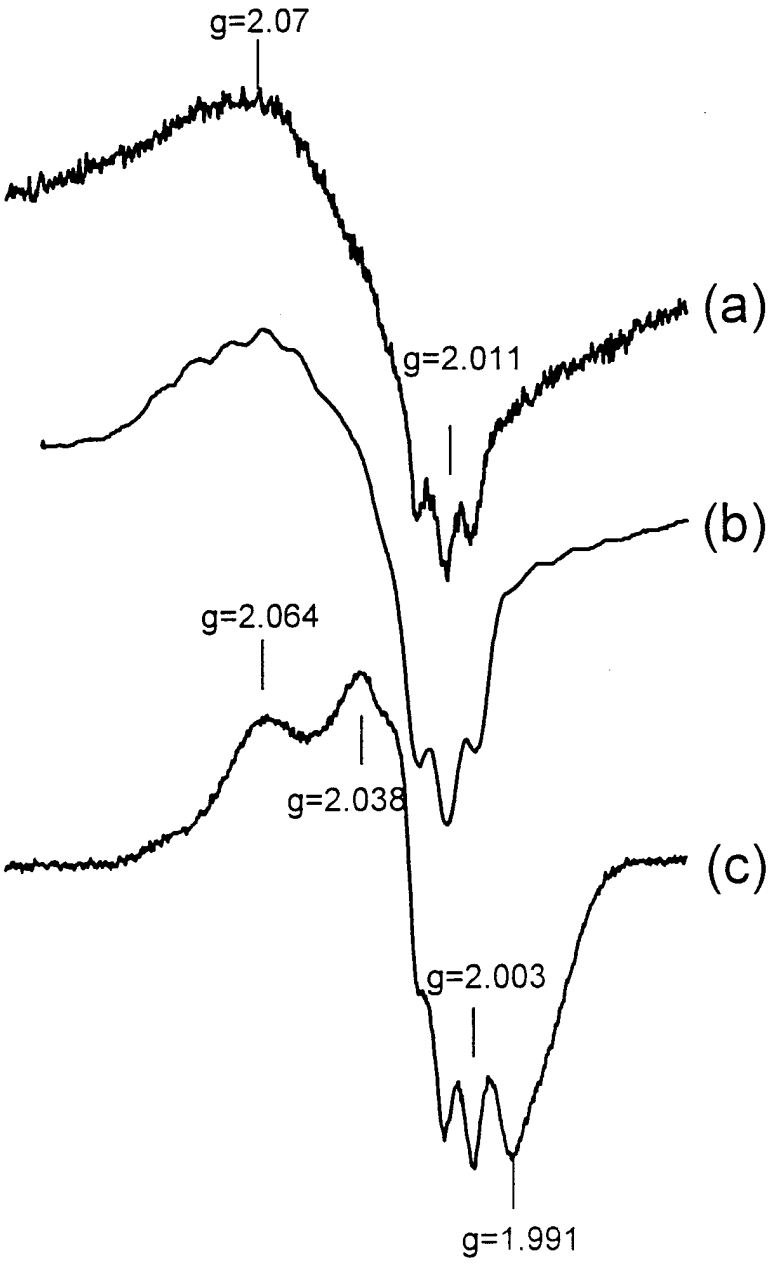

Fig. 1

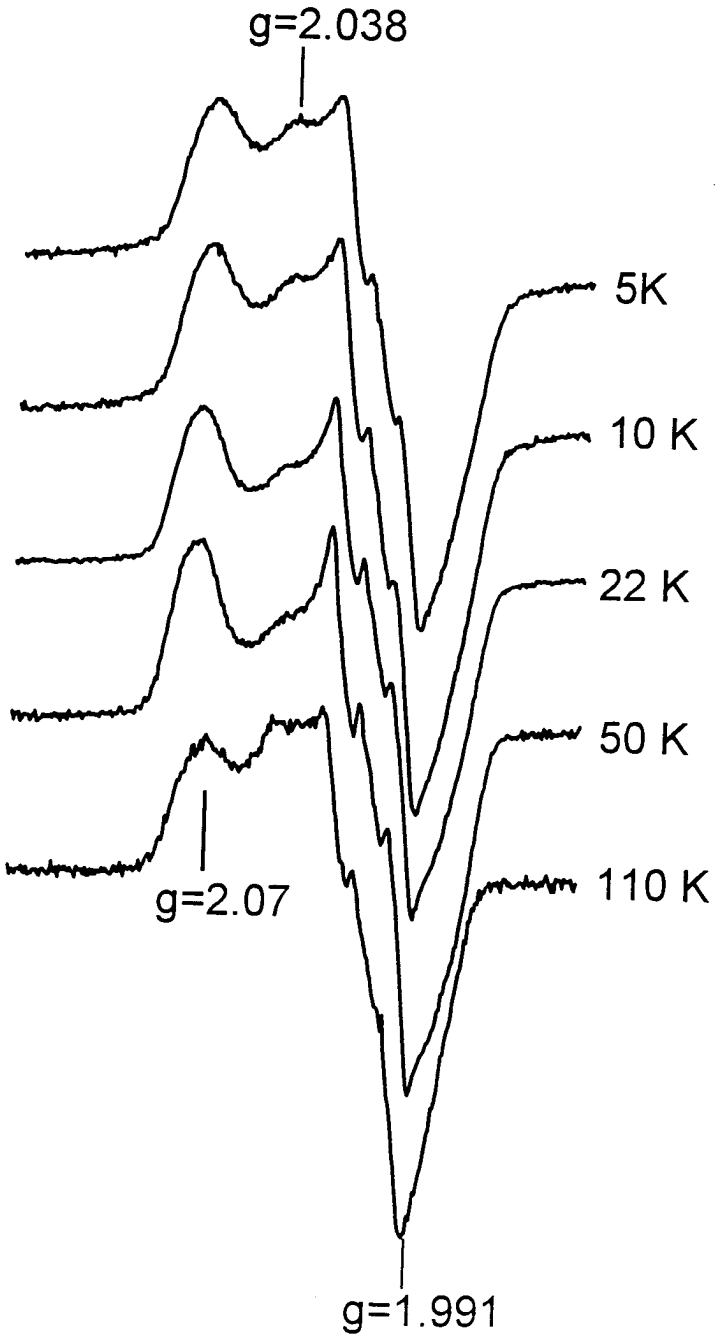

Fig. 2

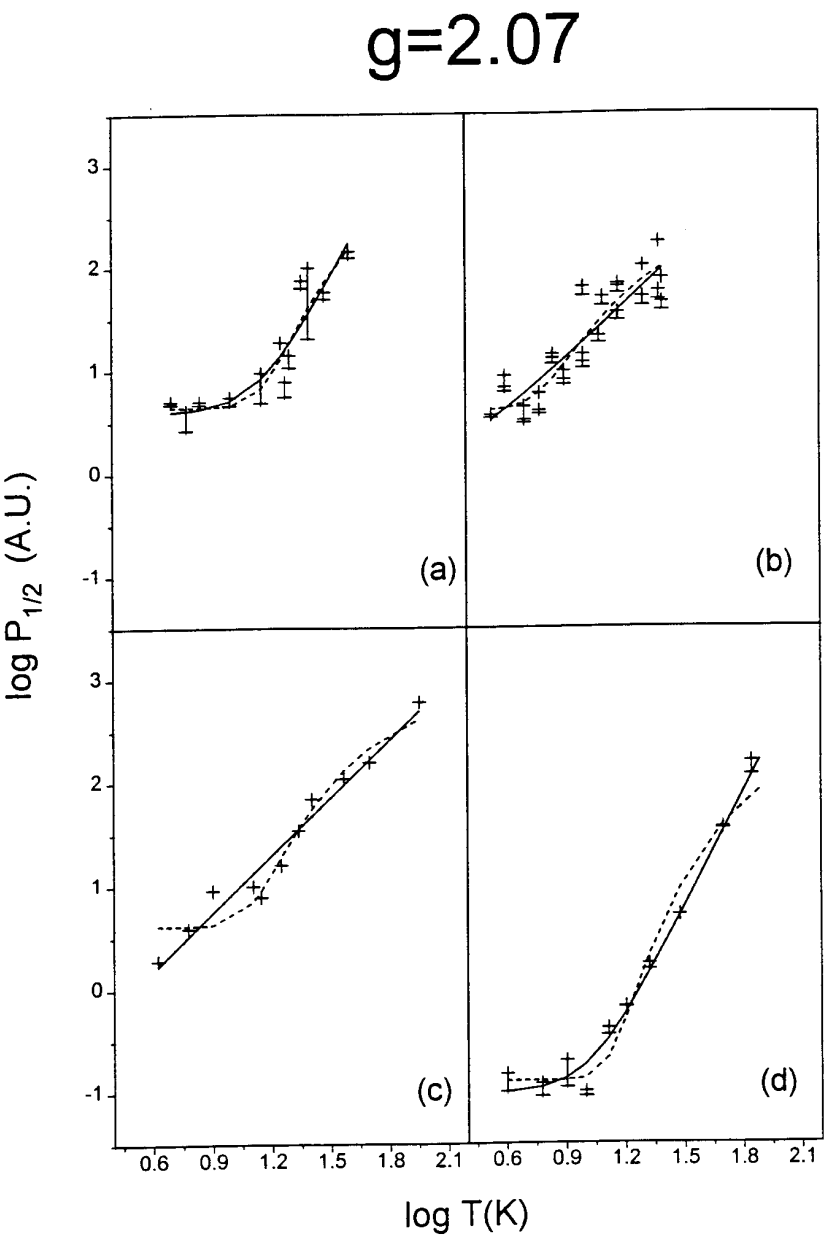

Fig. 3

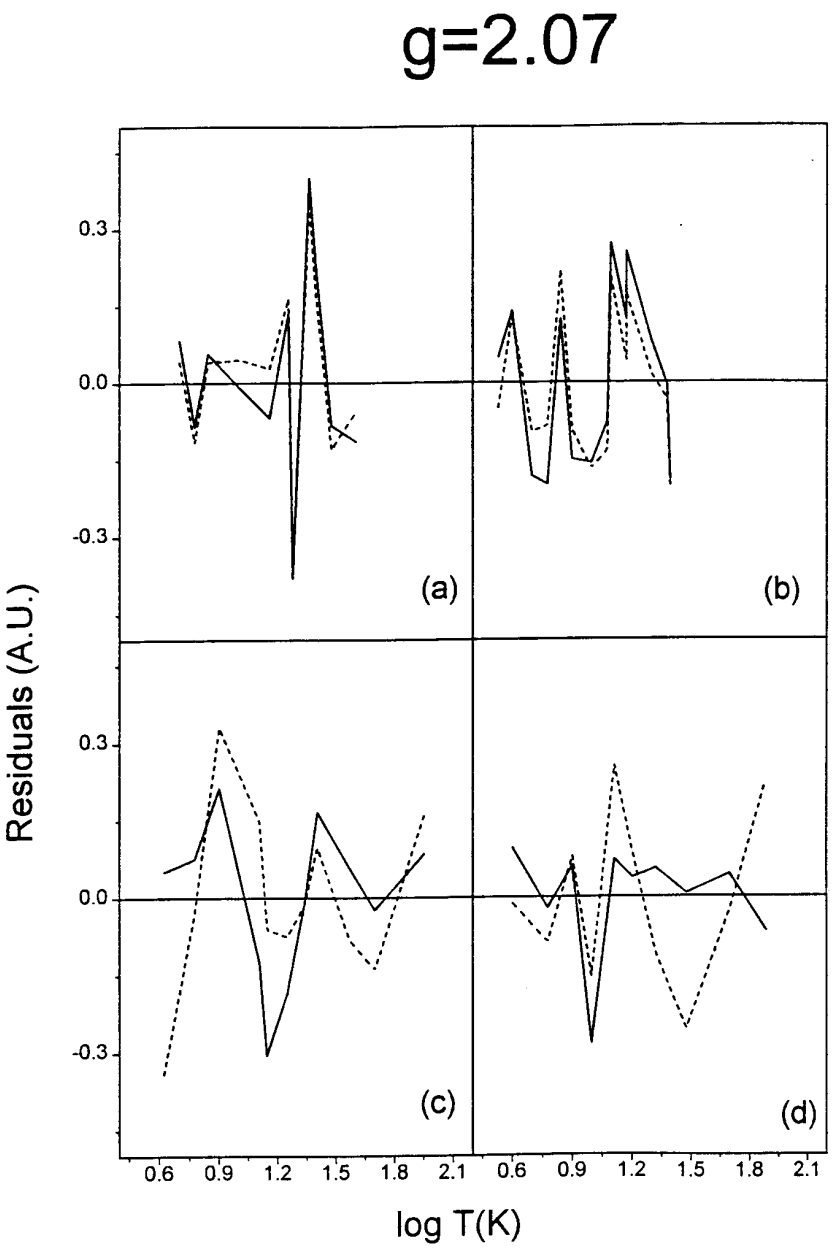

Fig. 4

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