Biophysical Chemistry 24 (1986) 319–325
Elsevier

BPC 01081

DISCRIMINATION OF TERTIARY AND QUATERNARY BOHR EFFECT IN THE O_2 BINDING OF *HELIX POMATIA* β-HEMOCYANIN

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Received 9th April 1986
Accepted 11th June 1986

Key words: Allostery; Respiratory protein; Proton release

Simultaneous determination of proton uptake and oxygen binding has been carried out on *Helix pomatia* β-hemocyanin under equilibrium conditions in the absence of buffer and at different initial pH values. Oxygen-binding isotherms of unbuffered *H. pomatia* β-hemocyanin, in the presence of phenol red as pH indicator, have been determined employing a thin-layer apparatus. Application of this very accurate technique allows monitoring of proton uptake (or release) coupled to O_2-binding also at extremes of saturation which are often difficult to explore and analyze. The data have been analyzed within the framework of the cooperon model (M. Brunori, M. Coletta and E. Di Cera, Biophys. Chem. 23 (1986) 215) and compared with those obtained in the presence of buffer. Comparison of pH changes with ligand binding of the T state over all the saturation range has allowed us to discriminate and obtain quantitative estimates of the Bohr protons associated with both oxygenation of the T state and quaternary allosteric transition; no protons are taken up or released during oxygenation of the R state. These results differ quantitatively from those obtained in the presence of buffer, which alters significantly the T state contribution to the overall Bohr effect.

1. Introduction

Hemocyanins, the respiratory proteins of a number of invertebrates, reversibly bind O_2 or CO at their active site. O_2 is bound cooperatively and marked positive or negative Bohr effects are often observed [1,2]; in contrast, binding of CO is essentially noncooperative and is not affected by pH [3–5]. As in the case of hemoglobins, the classical two-state allosteric model [6] is adequate but not sufficient to describe the cooperative behavior in the binding of O_2 by hemocyanins [7,8]. Although some progress has been made in describing O_2-binding curves by introducing the concept of 'functional constellations' [9], in such a model the Bohr effect can only be explained by the preferential binding of protons to one of the two quaternary states [6]. However, in the case of hemoglobins and hemocyanins, protons not only shift the equilibrium between the T and R state (quaternary-linked Bohr protons), but also affect the intrinsic functional properties of the state to which they preferentially bind [10]. This is shown, for example, by the fact that the position of the lower asymptote of the O_2-binding isotherms is pH-dependent, while the upper asymptote is invariant with pH [7,8,10,11].

Recently, oxygen equilibrium data of *Helix pomatia* β-hemocyanin have been successfully analyzed with a model which describes each functional constellation as being composed of smaller functional units, called 'cooperons' [12,13]. Such a model, although maintaining several of the features of the MWC model, incorporates the possi-

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bility of the coexistence of tertiary- and quaternary-linked heterotropic effects. Thus, in an attempt to dissect the relative contribution of the Bohr effect of *H. pomatia* β-hemocyanin, we have analyzed accurate O_2-binding curves of unbuffered β-hemocyanin determined in the presence of a pH indicator (phenol red) within the framework of the cooperon model [12,13]. Analysis of the data reveals that a relevant fraction of the Bohr protons (30–40%) is associated with oxygenation of the T state, while no protons are taken up or released on oxygenation of the R state. Although this feature was already foreseen from the binding isotherms in the presence of buffer, it should be mentioned that this is the first direct observation and quantitative analysis of a tertiary Bohr effect in the T state. Moreover, in view of the buffer effect on β-hemocyanin previously reported [14], the analysis has also allowed us to establish that the contribution of T state binding to the overall Bohr effect decreases significantly in the presence of buffer.

2. Materials and methods

H. pomatia β-hemocyanin, prepared and regenerated as described previously [7], was dialyzed against deionized and distilled water but containing 17 mM $CaCl_2$. Hemocyanin concentration is given in terms of O_2-binding equivalents, taking the value of 50 kDa as the molecular mass corresponding to one binding site. Phenol red (pK_a = 7.7) was obtained from Merck. In all experiments the molar ratio protein/dye was 1 : 1; under these conditions no binding of the dye to the protein was observed [15].

Oxygen equilibrium measurements were carried out by the thin-layer dilution method [16] in order to obtain accurate O_2-binding curves, especially at low and high saturation levels. Basically, the method consists of measuring the change in optical absorption of both protein and dye at successive stages of deoxygenation. The partial pressure of O_2 is adjusted in a stepwise manner by means of a precision dilution valve and may be constantly monitored by an oxygen electrode. At each oxygen dilution step the absorbance changes of the O_2-copper band at 365 nm (the isosbestic wavelength of the dye) and of the dye at 560 nm were simultaneously recorded with a Cary 219 spectrophotometer.

Unbuffered hemocyanin solution, equilibrated with pure O_2, was brought to the desired initial pH by addition of dilute HCl or NaOH. The concentration of hemocyanin employed for the thin-layer apparatus ranged between 0.6 and 1.0 mM (in binding sites), which is high enough to maintain a constant initial pH of the solution during transfer. In calculating proton uptake the observed value of absorbance at each stepwise dilution of O_2 binding was converted to ΔpH using the Henderson-Hasselbach equation:

$$\Delta pH = pK + \log \frac{[D^-] + [\Delta D]}{[HD] - [\Delta D]}$$

where [HD] and $[D^-]$ are the concentrations of the nonionized and ionized form of the dye, respectively; [ΔD], the decrease in [HD], was calculated from the change in dye absorbance. ΔpH was converted to ΔH^+ at each p_{O_2} dilution step by using the titration curve of hemocyanin plus dye. The ΔpH associated with complete O_2 saturation of protein was also measured potentiometrically on the same sample, following the procedure reported before [17].

3. Results

In view of the existence in *H. pomatia* β-hemocyanin of a buffer effect [14], discrimination between buffer-linked and O_2-linked Bohr effects requires O_2-binding isotherms in the absence of buffer and in the presence of a suitable dye (in this case phenol red) that allow monitoring of the proton-oxygen-linked pH changes. As shown in previous studies [7], this hemocyanin is characterized by a marked negative Bohr effect within the range pH 7.0–8.0. Thus, deoxygenation of an unbuffered solution of β-hemocyanin containing phenol red is associated with a decrease in absorbance both at the copper-oxygen band (365 nm) and at 560 nm, where the dye monitors the acidification linked to release of protons by the

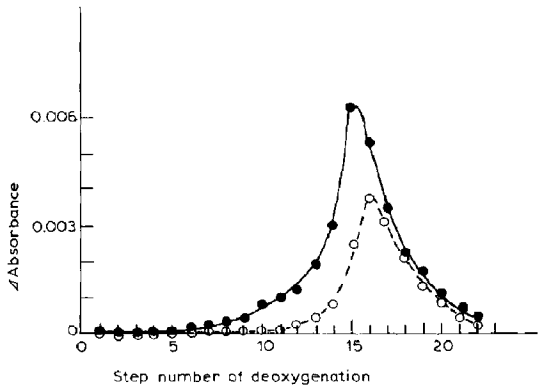

Fig. 1. Changes in optical absorbance of an unbuffered solution of *H. pomatia* β-hemocyanin and phenol red indicator following stepwise dilution of the O_2 partial pressure upon addition of N_2. The optical changes have been recorded at 365 nm (●———●), which monitors the O_2-copper complex of the protein, and at 560 nm (O------O) which monitors the dye absorbance. Initial pH of the solution was 7.2 at 25 °C.

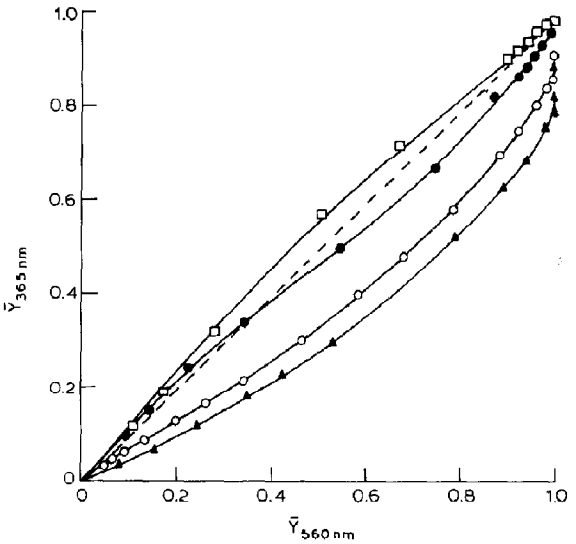

Fig. 2. Correlation between O_2 saturation of *H. pomatia* β-hemocyanin (recorded at 365 nm) and dye changes (measured at 560 nm). The continuous lines refer to experiments at different initial and final pH values, as follows: (□) pH 7.80–7.72, (●) pH 7.40–7.28, (○) pH 7.20–7.08, (▲) pH 7.10–7.04. The dashed line is the diagonal. Experimental conditions: protein and dye concentration were 800 μM, respectively, plus 17 mM $CaCl_2$ at 25 °C.

protein. Simultaneous recording at both wavelengths allows one to determine the O_2 saturation and the concomitant change in pH. A typical experiment performed with the thin-layer apparatus at an initial pH of 7.4 is represented in fig. 1, where the change in absorbance at both wavelengths is plotted against the number of stepwise dilutions of O_2 partial pressure [16]. These data clearly show that the first steps of deoxygenation (observed through changes in absorbance at 365 nm) are not associated with absorbance changes at the dye band (560 nm); as deoxygenation proceeds, the absorbance changes at the two wavelengths are almost linearly associated.

Fig. 2 shows the correlation between the two parameters observed starting at different initial pH values. It may be seen that at lower pH (7.10 and 7.20) this correlation is clearly nonlinear and O_2 saturation lags behind proton uptake at low O_2 pressures while uptake of protons during oxygenation is over before complete O_2 saturation. On the other hand, at pH 7.80 the uptake of protons lags somewhat behind O_2 binding and at intermediate pH (7.40) the overall curve is slightly sigmoidal. This nonlinear relationship between proton uptake and O_2 binding, observed at all pH values examined, implies a quantitatively different contribution of tertiary- and quaternary-linked proton uptake to the observed Bohr effect.

3.1. Binding curve analysis

The cooperon model employed here for the analysis has been quantitatively discussed elsewhere [12]: it postulates that a macromolecule is composed of m noninteracting functional constellations [9], each one being in allosteric equilibrium between two quaternary conformations. Each functional constellation is formed of z functional units, called 'cooperons'. Therefore, assuming a dimeric cooperon, the binding polynomial [18] of a functional constellation is

$$P(x) = \left[L_0\left(1 + 2K_T x + \delta_T K_T^2 x^2\right)^z + \left(1 + 2K_R x + \delta_R K_R^2 x^2\right)^z\right]/(1 + L_0) \quad (1)$$

where x is the ligand activity, K_T and K_R the observed binding constants in the two quaternary states, $L_0 (= [T_0/R_0])$ the allosteric constant, and

δ_T and δ_R interaction constants between the subunits in a cooperon for each quaternary state ($\delta > 1$, positive cooperativity; $\delta < 1$, negative cooperativity; $\delta = 1$, no cooperativity).

These parameters are linked through the stabilization factors, $\tau_{i,j}$, and $\rho_{i,j}$ ($i = 0$ or 1, $j = 0$ or 1), characteristic for each quaternary structure (T and R), and refer to the tertiary state of the two interacting subunits of the cooperon. Thus,

$$L_0 = (\tau_{00}/\rho_{00})^z;$$

$$K_T = K_t(\tau_{01}/\tau_{00});\ K_R = K_r(\rho_{01}/\rho_{00});$$

$$\delta_T = (\tau_{11}\tau_{00}/\tau_{01}^2);\ \delta_R = (\rho_{11}\rho_{00}/\rho_{01}^2);$$

where K_t and K_r are the intrinsic binding constants of subunits in the two quaternary states and 0 and 1 denote unliganded and liganded, respectively. The five parameters appearing in eq. 1 may be affected by heterotropic effectors, which alter the stabilization factors; therefore pure tertiary- or quaternary-linked, as well as mixed effects, are possible [12]. In the analysis of the data described in this paper it was found that $\delta_R = 1$; thus, by reference to eq. 1 the number of parameters necessary to describe the ligand-binding process corresponds to four.

In the analysis of data, we have first determined the effect of the pH on the model parameters obtained by fitting the O_2-binding isotherms in a buffered solution (10 mM Tris-HCl) assuming a functional constellation of eight dimeric cooperons ($z = 8$). The pH dependence is reported in fig. 3a and b, where it appears that pH affects not only the quaternary constant L_0 but also the two tertiary parameters, namely, K_T and δ_T. The theoretical curves of these parameters have been described quantitatively employing the following set of equations:

$$K_T = \tilde{K}_T(1 + K_{\tau_{01}}h)^n/(1 + K_{\tau_{00}}h)^p \qquad (2a)$$

$$\delta_T = \tilde{\delta}_T(1 + K_{\tau_{11}}h)^s(1 + K_{\tau_{00}}h)^p/(1 + K_{\tau_{01}}h)^{2n} \qquad (2b)$$

$$L_0 = \tilde{L}_0\left((1 + K_{\tau_{00}}h)^p/(1 + K_{\rho_{00}}h)^r\right) \qquad (2c)$$

where h is the proton activity, K terms the values of the proton-binding constants associated to the different configurations of the cooperon, $\tilde{K}_T$, $\tilde{\delta}_T$, $\tilde{L}_0$ the asymptotic values of the parameters and n, p, r, s the number of independent titratable groups linked to the corresponding configuration. In the first column of table 1 the pK_a values of the different classes of titratable groups are reported together with the number of groups for each class. Although we are not in a position to determine unequivocally the number of titratable groups involved and to assign the pK_a values to specific groups, we deem these numbers (8 for the T state and 16 for the R state) reasonable; this also in view of the fact that the cooperon (corresponding to the ~ 100 kDa fragment) contains 44 histidines [19], even though it does not necessarily mean that the eight groups are all histidines. In the absence of buffer, the analysis of binding curves has been focussed on the data set around pH 7.4 (see fig. 2) since this pH value is close to the pK_a of the dye and the Bohr effect is maximal, both conditions contributing to enhance the proton-linked signal associated with deoxygenation of the protein. Since the actual pH at each saturation level is obtained from the absorbance of the pH indicator, the value of $\overline{Y}_T$ to reproduce the curve at pH 7.4 of fig. 2 can be determined (at a given pH and p_{O_2}) by eq. 1 using the values of L_0, δ_T and K_T derived from buffered O_2-binding curves at the corresponding pH value (see eqs. 2). However, a satisfactory simulation of the experimental data set at pH 7.4 of unbuffered β-hemocyanin has only been possible by modification of the pK_a of the titratable groups reported in table 1, leaving unchanged the asymptotic values of the parameters determined under buffered conditions. In general, simulation of binding curves carried out at different initial pH values has always required a small increase of the pK_a values. However, this modification became smaller at lower initial pH values of the O_2-binding curve.

The pK_a values used in the simulation at pH 7.4 are compared in table 1 with those obtained from the fit of the corresponding buffered O_2-binding curves; the observed difference has been attributed to the effect of buffer on the proton-oxygen linkage in *H. pomatia* β-hemocyanin [14]; this difference disappears at lower pH, where the buffer is predominantly in the protonated form.

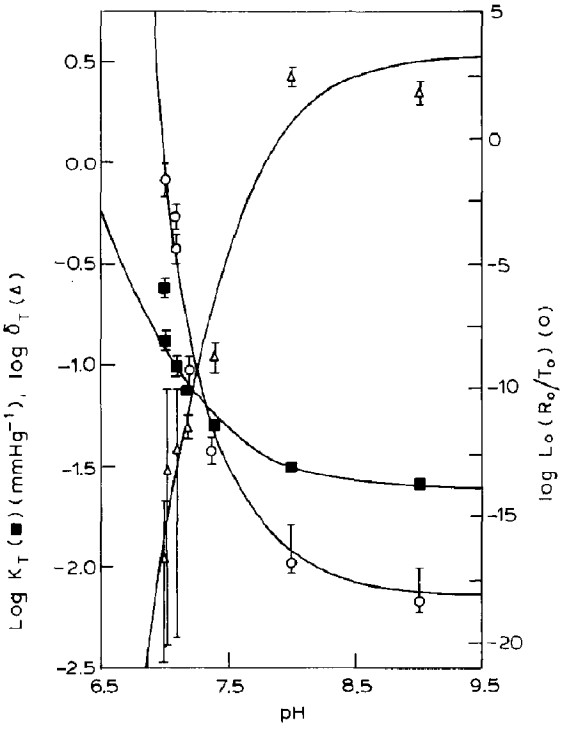

Fig. 3. pH dependence of the parameters for the cooperon model applied to *H. pomatia* β-hemocyanin in the presence of 10 mM Tris-HCl. δ_T (Δ), K_T (■) and L_0 (○). The continuous lines have been obtained using eqs. 2a–c and the pK_a values are reported in the first column of table 1.

Using the pK_a values reported in the second column of table 1 and eqs. 1 and 2 it is possible to sort out quantitatively the tertiary and quaternary contribution to the Bohr effect of the oxygen-proton linkage, since the percentage of oxygenation

Table 1

pK_a values of the different classes of titratable groups of *H. pomatia* β-hemocyanin determined by fitting of buffered O_2-binding curves and simulation of unbuffered O_2-binding curves

The number of titratable groups per cooperon unit used in the simulations are reported in parentheses.

	Buffered solution	Unbuffered solution
$pK_{\tau_{00}}$	6.64 (8)	6.82 (8)
$pK_{\tau_{01}}$	6.88 (8)	7.07 (8)
$pK_{\tau_{11}}$	6.00 (8)	7.20 (8)
$pK_{\rho_{00}}$	6.74 (16)	6.92 (16)

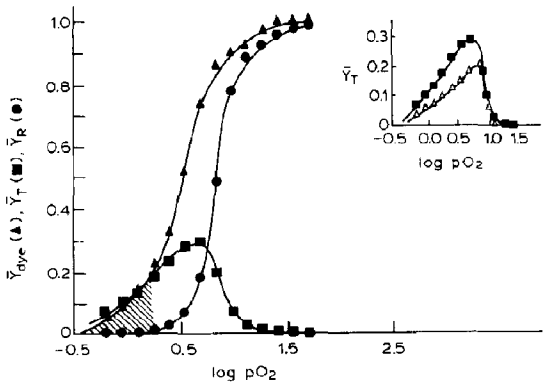

Fig. 4. Fractional pH changes (▲———▲), fraction of oxygenated T state ($\bar{Y}_T$) (■———■) and R state ($\bar{Y}_R$) (●———●). $\bar{Y}_T$ and $\bar{Y}_R$ have been calculated from eq. 3 (see footnote, below) and eq. 2 using the pH values reported in the second column of table 1. The inset compares $\bar{Y}_T$ of unbuffered (■———■) and buffered (10 mM Tris-HCl) (Δ———Δ). The shaded area indicates the Bohr effect due to oxygenation of the protein in the T state.

of the macromolecule ($\bar{Y}$) and of the T state ($\bar{Y}_T$) can both be calculated * at every pH and p_{O_2} and related to the extent of the dye change along the binding curve, as shown in fig. 4. In the inset to fig. 4, $\bar{Y}_T$ values determined for buffered and unbuffered O_2-binding curves are compared.

4. Discussion

Correlation of proton uptake with O_2 binding represents a stringent test for any allosteric model used to describe the cooperative binding behaviour of O_2-carrying proteins. Analysis of proton release in hemoglobin has provided, with some exceptions [20], contradictory results [21,22]; this is probably due to the fact that in hemoglobin the two allosteric states are both significantly popu-

* $\bar{Y}_T$ has been calculated at each p_{O_2} and pH by using the equation:

$$\bar{Y}_T = \frac{L_0 K_T x (1+\delta_T K_T x)(1+2K_T x+\delta_T K_T^2 x^2)^7}{L_0(1+2K_T x+\delta_T K_T^2 x^2)^8+(1+K_R x)^{16}}$$

where the parameters are those defined in eq. 1 and their pH dependence was calculated using eq. 2a.

lated throughout the binding curve and can be primarily detected only at the very extremes of the saturation isotherm. On the other hand, in the case of *H. pomatia* β-hemocyanin, the shape of the O_2-binding curve indicates that the two quaternary states of the macromolecule can be studied individually over an appreciable range of O_2 pressures, since the conformational transition takes place within a relatively narrow range of saturations. Thus, combination of a particular protein such as *H. pomatia* β-hemocyanin with methods involving high-precision determination of O_2 binding and pH changes, especially at low and high saturation levels, has proven to be of crucial importance in discriminating between tertiary and quaternary Bohr effect contributions.

The data reported in fig. 1 clearly show that at pH 7.4 during the first steps of deoxygenation the pH of the solution remains constant, while once the quaternary conformational transition takes place (before the top of the curve [16]) an appreciable decrease of fractional O_2 saturation and of pH take place simultaneously. This result indicates that virtually no Bohr effect is associated with O_2 binding to the R state, even in the absence of buffer, while a large release of Bohr protons is linked to the quaternary switch-over and deoxygenation of the T state. This is confirmed by the results of the analysis reported in fig. 4 where the fractional changes of the dye are correlated with $\overline{Y}_T$ and $\overline{Y}_R$. Moreover, the fractional pH change in the shaded region relates entirely to the $\overline{Y}_T$ curve, indicating unequivocally the existence of a (local) Bohr effect within this state. In view of the fact that O_2 binding to the T state continues after the appearance of an appreciable amount of protein in the R state, approx. 30–40% of the observed Bohr effect in the absence of buffer can be related to the binding of O_2 in the T quaternary state.

The results reported above represent the first quantitative correlation between proton release and oxygenation for a tertiary Bohr effect. In fact, previous kinetic and equilibrium experiments carried out on fish or human hemoglobin [11,23] pointed out the great difficulty of studying the intrinsic properties of the T and R states in a system which displays cooperative ligand binding.

Application of the cooperon model has made it possible to describe quantitatively the binding data and to quantitate the buffer effect on *H. pomatia* β-hemocyanin [15]. It is found that in buffered solution the fraction of oxygenated T state ($\overline{Y}_T$) is lower (see inset to fig. 4), and thus its relative contribution to the overall Bohr effect is about half of that expressed in the absence of buffer, as shown by the present analysis. A closer inspection of the pK_a values reported in table 1 shows that the presence of Tris-HCl buffer (10 mM) brings about a $\Delta pK_a = 0.17 \pm 0.01$ for $pK_{\tau_{00}}$, $pK_{\tau_{01}}$ and $pK_{\rho_{00}}$, whereas for $pK_{\tau_{11}}$ the $\Delta pK_a = 1.2$. Thus, the net effect of the buffer seems to be a marked decrease in affinity of the protons for the fully liganded form of the dimeric cooperon in the T conformation, since proton binding to the T state is negatively cooperative in the presence of buffer ($pK_{\tau_{11}} < pK_{\tau_{01}}$) and positively cooperative in its absence ($pK_{\tau_{11}} > pK_{\tau_{01}}$). Moreover, the increased number of titratable groups in the R state, from 8 to 16 (see table 1), reflects a pK_a shift of some groups on allosteric transition. This suggests that in the presence of buffer, stabilization of the R state at lower pH may be due to a larger number of accessible groups rather than to a higher proton affinity of the R state. Furthermore, the possibility of describing the behaviour of unbuffered β-hemocyanin by simply shifting the pK_a values of the different cooperon configurations without varying the asymptotic values suggests that the observed buffer effect is mediated by protons. Thus, the buffer O_2 linkage reported could be defined as a pseudo-linkage [24], i.e., a consequence of the buffer-proton and proton-O_2 linkages. This possibility should be always borne in mind when dealing with different heterotropic effectors in allosteric macromolecules, and indicates that with complex cooperative systems it is difficult to find a really 'innocent' buffer.

In conclusion, this analysis agrees satisfactorily with the knowledge that in several oxygen carriers there is a pH effect on the low asymptote of the O_2 isotherms; it also suggests that the previous statement of a Bohr effect wholly associated with the quaternary transition, as obtained on the basis of kinetic experiments [15], was an oversimplification likely due to the difficulties intrinsic to a

quantitative comparison of the static and kinetic absorbance changes of the unbuffered system, without however affecting the dynamic conclusions reached by analysis of the stopped-flow data [15]. Moreover, the cooperon model [12,13], even in its simplest version, appeared perfectly suitable to describe quantitatively the interplay among different ligands in a multimeric protein. The mechanism of the Bohr effect resulting from such an investigation offers a satisfactory picture of the whole process and a detailed description of the various linkages involved.

Acknowledgements

The authors express their thanks to Professor S.J. Gill for providing a thin-layer apparatus. This work has been partially supported by a grant from the M.P.I. (Italy) to M.B., and by Nato Grant N.132.82.

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