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Subunit hybridization studies of partially ligated cyanomethemoglobins using a cryogenic method

Evidence for three allosteric states

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Reaction of tetrameric hemoglobin with ligands at the four heme sites yields nine species that have structurally unique combinations of ligated and unligated subunits. Using hemoglobins where the ligated subunits contain cyanomethemoglobin, Smith and Ackers studied the dimer-tetramer assembly reactions in all nine of the partially ligated species (F.R. Smith and G.K. Ackers, Proc. Natl. Acad. Sci. U.S.A. 82 (1985) 5347). They found a third assembly free energy in addition to those of unligated hemoglobin and fully ligated cyanomethemoglobin. The observed distribution of the three assembly free energies among the ten species was found to be incompatible with the two-state mechanism of allosteric control (J. Monod, J. Wyman and J.P. Changeaux, J. Mol. Biol. 12 (1965) 81). The results indicated a mechanism of 'combinatorial switching' in which the binding free energies per site change with configuration of occupied sites and not just their number. In this study, we have confirmed the existence of three assembly free energies among the ten ligation species using a cryogenic method (M. Perrella and L. Rossi-Bernardi, Methods Enzymol. 76 (1981) 133). For one of the species we find a different free energy assignment from that reported by Smith and Ackers; for all other species we observe the same assignments as in earlier work. The revised distribution also requires a 'combinatorial' mechanism of allosteric switching among the three states.

1. Introduction

Human hemoglobin has been studied intensively for several decades as a prototype for understanding how macromolecular assemblies can utilize subunit interactions to carry out regulation of biological functions. Most hemoglobin research has been based upon, or utilized, the allosteric model of Monod et al. [1] in which the molecule exhibits only two allosteric states, i.e., two molecular structures (with different ligand affinities) which participate in a labile conformational equilibrium ($R \leftrightarrow T$). This model has been found capable of describing a large body of experimental results on hemoglobin as well as other cooperative systems.

In 1985, Smith and Ackers [2] reported experimental evidence for the existence of a third allosteric state in the process of ligating deoxyhemoglobin. Using a combination of kinetic methods and analytical gel chromatography, they studied the subunit dissociation and assembly (dimers to tetramers) of the ten tetrameric species, representing all structurally unique combinations

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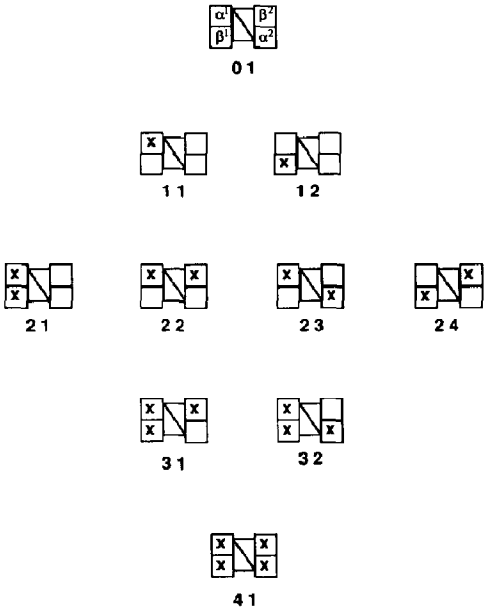


Fig. 1. Topographic representation of the ten ligation states of tetrameric hemoglobin. The index *ij* denotes the particular species *j* among those with *i* ligands bound ($i = 0$–4, $j = 1$–4). Ordering of species with respect to *j* is arbitrary. Subunit positions are shown in species 01. Intersubunit contacts are denoted by solid lines. Assembly free energies discussed in this paper apply to formation of each species *ij* from its constituent dimers shown here as left and right of each tetramer.

of ligated and unligated subunits (see fig. 1). These data provided the unexpected result that three distinct free energies of dimer-tetramer assembly exist among the ten species. The lowest free energy (−14.3 kcal/mol) was found uniquely for deoxyhemoglobin (species 01, fig. 1) and the highest value (−8.5 kcal/mol) was observed for five of the species: the fully ligated tetramer (species 41), the two triply ligated species (31 and 32 of fig. 1) and the two symmetric doubly ligated species (i.e., 23 and 24). A third distinct free energy (−11.4 kcal/mol) was found for the two singly ligated species (11 and 12) and the two asymmetric doubly ligated species (21 and 22), i.e., with ligands on the α^1 and β^1 subunits or on the α^1 and β^2 subunits, respectively. Assuming the dissociated dimeric species to be noncooperative (i.e., to bind successive ligands with the same intrinsic affinity) and the ligand affinities of α and β subunits within the tetramer to be similar, Smith and Ackers [2,3] showed that the observed distribution of assembly free energies was incompatible with the two-state mechanism of allosteric control. The same result was obtained with two other ligand-model systems in which tetrameric structures included subunits where the heme iron had been replaced with manganese [4]. Consistent with these results is the recent finding that, among doubly ligated tetramers, the symmetric species (i.e., 23 and 24, fig. 1) and asymmetric species (i.e., 21 and 22, fig. 1) have very different affinities for carbon monoxide, as determined in studies of the equilibrium interaction using cryogenic techniques [5] and in investigations of the rates of CO dissociation from hemoglobin-CO intermediate species [6].

Because of the potential significance of a third allosteric state to the mechanistic understanding of hemoglobin, we have carried out a study of the cyanomethemoglobin system by an independent method. In this paper, we report experimental results on the cyanomethemoglobin intermediates using cryogenic techniques developed by Perrella and co-workers [7–9]. We have confirmed the basic finding of Smith and Ackers [2–4] that there are three distinct free energies of assembly for the ten 'ligation states'. A discrepancy was observed with regard to assignment of the assembly free energy for one of the species, i.e., species 22 (fig. 1). The likely origins of this discrepancy are discussed.

2. Materials and methods

2.1. Preparation of hemoglobins

Hemoglobins A_0 and C were prepared by ion-exchange chromatography from red cell lysates. Partially oxidized, symmetrical hemoglobin hybrids were prepared by chromatography of oxyhemoglobin samples 50% oxidized by ferricyanide at pH 6.8 for 2 h at 20°C ($\alpha_2^+ \beta_2^{O_2}$) and for 1 min at 0°C ($\alpha_2^{O_2} \beta_2^+$) before removal of ferrocyanide by gel chromatography.

Before storage in liquid N_2, the oxyhemoglobin samples were equilibrated in 0.1 M Tris-HCl, 0.1 M NaCl, 0.1 mM EDTA, 0.1 mM KCN. Catalase

and superoxide dismutase were routinely added to the protein solution for protection against autooxidation. Samples of the purified hemoglobin were occasionally stored under CO. Removal of CO was carried out by rotating 20–30 ml of protein solution (4 g/dl) in a 2 l cylindrical glass bottle flushed with oxygen, under 100 W light at 0 °C for approx. 30 min.

2.2. Anaerobic incubation of 1 : 1 mixtures of symmetrical parent species

Deoxygenation of hemoglobin samples (0.5–1.0 ml at 5–6 g/dl) was carried out in a glass tonometer of the Radiometer type (Radiometer, Copenhagen) for 1–1.5 h using an N_2 stream purged through an alkaline solution of dithionite. Before deoxygenation more catalase and superoxide dismutase were added to the sample and its pH was readjusted to pH 7.4 with Tris-HCl buffer (pH 1.5). Excess cyanide (1 mM) was added to samples in cyanomethemoglobin form.

The deoxygenated samples were injected using gas-tight Hamilton syringes into a 5 ml glass vial purged with N_2 and sealed with a 5 mm thick silicone rubber cap under a positive N_2 pressure. A 1 : 1 mixture of parent symmetrical hemoglobin species (one parent being hemoglobin A_0 and the other hemoglobin C) was then incubated at 21.5 °C for times varying from 4 min to 80 h by placing the vial in a glass cylinder continuously flushed with N_2 and kept in a thermostatted water bath. At the end of the incubation period the vial containing the mixture, equilibrated (partially or completely) under anaerobic conditions, was flushed with CO and the analysis of the mixture was carried out as described below.

2.3. Quenching of hemoglobin samples at subzero temperature

Hemoglobins A_0 and C, differing by a single surface charge (HbC: β6 Glu–Lys), have essentially the same free energies of assembly in both their unligated and fully ligated forms [10]. A mixture of parent molecules and their hybrid can be separated by cryogenic electrophoretic methods, after blocking the dimer exchange reactions in the aqueous medium by quenching the solution in a hydro-organic solvent at subzero temperature [6]. Anaerobic samples (0.1 ml) containing the parent species and their hybrid were injected into a cryogenic buffer (0.8 ml) (20 mM Tris-HCl, 2 mM KCN buffer, pH 8.0, diluted with an equal volume of ethylene glycol), saturated with CO at −30 °C and mechanically shaken [8]. Under these conditions, the deoxyhemoglobins become CO bound. This procedure ensures that the analysis of the mixture by subzero temperature electrophoresis is carried out on hemoglobin species having the same stability, whether equilibrated anaerobically or during exposure to CO.

2.4. Subzero temperature gel electrophoresis

Previously described procedures were followed [6,8] with some modifications. Electrophoretic separations were carried out on gel tubes made of copolymers of ethylacrylate and acrylamide (T = 5.24%) with methylenebisacrylamide as the crosslinker (C = 2.64%). The ethylacrylate molar fraction was 0.31. The electrode buffer was 137 mM glycine-17 mM Tris, 2 mM KCN (pH 8.5) at 20 °C, diluted with ethylene glycol (30%, v/v) and methanol (20%, v/v).

The gels were loaded with 10 μl of the quenched hemoglobin mixture (0.5–0.7 g/dl). Electrophoresis was carried out at −25 °C and 300 V (about 0.1 mA/tube) for 3–4 h. Hemoglobin A_0 moved approx. 1.8 cm and was separated from hemoglobin C by approx. 6 mm. The hybrid species migrated halfway between the parents.

2.5. Determination of the relative amounts of parent species and hybrids

At the end of the electrophoretic run, the gels (usually five) were slipped out of the glass tubes and each was cut into three equally sized slices containing the parent species and their hybrid, respectively. The slices containing the same component were suspended in 2 ml of 50 mM sodium phosphate, 50 mM NaCl, 0.05% NaN_3 (pH 7.5) for 24 h to elute the protein, which was assayed by the pyridine hemochromogen method [11]. Pyridine (0.5 ml) was added to each suspension that

was kept in the dark [12] for no longer than 10 min before being transferred to a Thunberg cuvette containing solid dithionite (12 mg). Stable readings at 418 nm were attained within 1 min.

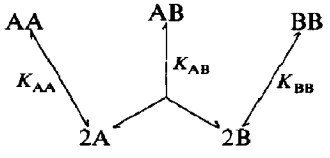
The method was calibrated using a standard hemoglobin solution. Known samples were diluted in the same buffer as used for elution of the protein from gel slices.

2.6. Controls

Controls were run to check that: (a) no hybridization of parent species occurs during the injection of the aqueous samples into the cryogenic buffer at $-30\,^{\circ}C$, during the transfer of the 10 μl sample onto the gels during the time required for hemoglobin resolution; (b) no significant dissociation of asymmetrical hybrids in the ligated state occurs during the electrophoretic separation from the parent species. For this purpose, samples (0.05 ml) of each parent molecule were injected into the quenching medium at $-30\,^{\circ}C$. No hybrid formation was detected after the electrophoretic separation under the usual conditions. Mixtures of parent species and their hybrid in the carbonmonoxy- or cyanomethemoglobin form were separated by electrophoresis for times varying from 3 to 6 h. No significant deviation from the binomial (1 : 2 : 1) distribution was observed (see section 2.7).

2.7. Estimation of assembly free energies

The cryogenic technique allows one to calculate the free energy of the asymmetrical hybrid only when the free energies of the symmetrical parent species are known via independent determination. The general reaction scheme for these hybridization experiments is depicted below.



The experimentally resolved concentration distribution is related to the three intrinsic equilibrium constants of dimer-tetramer reaction by the mass law relationship:

$$\frac{[AB]}{\sqrt{[AA][BB]}} = \frac{2K_{AB}}{\sqrt{K_{AA}K_{BB}}} \tag{1}$$

Equilibrium constants on the right bear subscripts corresponding to the tetrameric species whose equilibrium concentrations are represented in brackets on the left. The numerical value of 2 is a statistical factor accounting for the two ways of forming the hybrid AB *. It follows from eq. 1 that the concentration of hybrid in a 1 : 1 mixture of parent molecules conforms to the binomial 1 : 2 : 1 distribution only if the free energy of formation of the hybrid is the mean of the free energies of formation of the parents ($\Delta G = -RT \ln K$). In all other cases, the concentration of the hybrid differs from twice that of each parent and is governed by the relative values of the free energies of the three reactions. While this method does not by itself permit one to determine the three assembly free energies, it has the important virtue of allowing the study of the molecular system under true thermodynamic equilibrium. Moreover, when K_{AA} and K_{BB} can be determined independently (e.g., by kinetic methods or analytical gel chromatography) K_{AB} follows directly from eq. 1 and the data.

The free energy of hybrid assembly ΔG_{AB} may differ from the mean value of the parent species by an amount δG:

$$\delta G = \tfrac{1}{2}(\Delta G_{AA} + \Delta G_{BB}) - \Delta G_{AB} \tag{2}$$

From eqs. 1 and 2, we observe

$$\delta G = RT \ln \frac{f_{AB}}{2(f_{AA}f_{BB})^{1/2}} \tag{3}$$

where f_{AB}, f_{AA} and f_{BB} are percentages of the respective species at equilibrium as determined by the cryogenic experiment. This 'deviation free en-

* With slight rearrangement of eq. 1:

$$\frac{[AB]}{K_{AB}} = 2\sqrt{\frac{[AA]}{K_{AA}} \cdot \frac{[BB]}{K_{BB}}}$$

We note that the hybrid concentration [AB] scaled to its own formation constant K_{AB} is twice the geometric mean of the corresponding values for the parent species.

ergy,' δG, is the energetic quantity determined directly by the cryogenic technique. In combination with independent determinations of the assembly free energies for the 'parent' species, (i.e., species 01, 23, 24 and 41; fig. 1) it provides a means of estimating free energies for the remaining intermediates (species 11, 12, 21, 22, 31, and 32).

3. Results and discussion

The experimental strategy followed throughout this work consisted of equilibrating at 21°C and pH 7.4, for times up to 80 h under anaerobic conditions, approx. 1 : 1 mixtures of hemoglobins A_0 and C in different, symmetrical states of ligation.

During the equilibration and after the attainment of equilibrium, samples of the mixture were collected for analysis by the cryogenic technique. The remaining mixture was then exposed to carbon monoxide to allow the concentration of the parent and hybrid species to attain the distribution pertaining to the fully ligated states of these species. This procedure ensured that species composition differences of little as 2% of the total, which were observed for the concentrations of some asymmetrical hybrids under the anaerobic and the ligated conditions, could be assigned to energetic differences of the species in the two states and not to artifacts of the methodology.

Results of these analyses are shown in figs. 2 and 3, and table 1. Fig. 2 shows a plot of the concentrations of the 'species 21' hybrid ($\alpha^{CN}\beta_c^{CN}$) ($\alpha^{deoxy}\beta^{deoxy}$) and the parent species, cyanomethemoglobin C and deoxyhemoglobin A_0, vs. incubation time prior to quenching in the cryosolvent. A distinctly nonbinomial equilibrium distribution of the hybrid and its parent species was reached in about 40 h, and maintained thereafter (i.e., in a binomial distribution the fraction of hybrid would be 0.5 at equilibrium). Upon exposure to carbon monoxide the mixture redistributed to a binomial (1 : 2 : 1) distribution (see table 1). The observed deviation from 'binomiality' for the deoxy system leads, via eq. 3, to an apparent 'non-additivity' of 0.15 kcal (table 1). The constant plateau value of 43.5% for the hybrid, and its

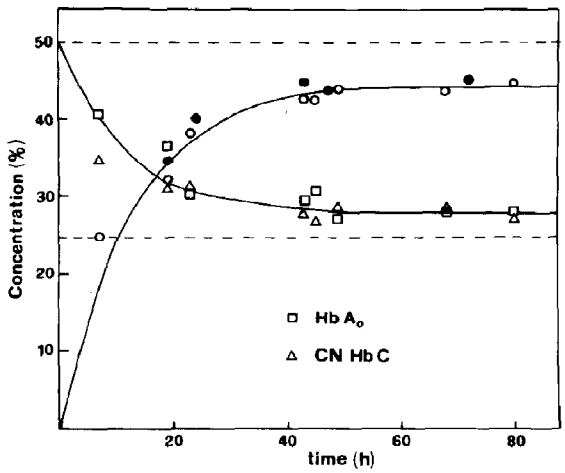


Fig. 2. Concentration of species '21' hybrid ($\alpha^{CN}\beta_c^{CN}$) ($\alpha^{deoxy}\beta^{deoxy}$) and the parent species (cyanomethemoglobin C and deoxyhemoglobin A_0) vs. incubation time prior to quenching in the cryosolvent. Open circles show the time course for appearance of the hybrid (footnote a, table 1). Filled circles show the corresponding time course for species 21 (footnote b, table 1) when the parent molecules are cyanomethemoglobin A_0 and unligated hemoglobin C.

shift, upon complete ligation, to 49.6% are a strong indication of the integrity and sensitivity of the experimental technique employed.

The amount of hybrid formed in this mixture after only 4 min of incubation was in the range of 2–4% of the total. This indicates the presence of

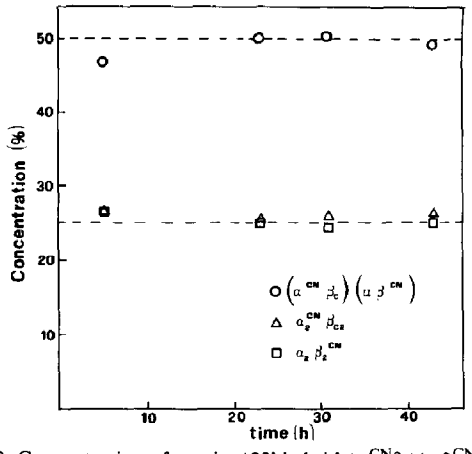


Fig. 3. Concentration of species '22' hybrid ($\alpha^{CN}\beta_c$)($\alpha\beta^{CN}$) vs. incubation time prior to quenching in the cryosolvent, along with the two parent species.

Table 1

Equilibrium concentrations of parent species (A_0 and C) in different ligation states (ij) and their hybrid (Y) under deoxy conditions and in the presence of carbon monoxide

Results in columns (1)–(3) refer to: (1) cryogenic results (the present study); (2) free energies of hybrid formation calculated using eq. 2 taking the δG values from cryogenic experiments in combination with free energies of the parent species given in ref. 2; (3) free energies from ref. 2. Experimental precision of results: ±0.5%.

Ligation state (ij)	Deoxy						Ligated			
	Species			(1) δG (kcal)	(2) ΔG_{AB} (kcal)	(3) ΔG_{AB} (kcal)	Species			δG (kcal)
	A_0	C	Y				A_0	C	Y	
01	–	–	–	–	–	−14.4	–	–	–	–
11	25.6	26.8	47.6	−0.056	−11.4	−11.5	24.7	25.2	50.1	0.002
12	27.4	26.6	45.9	−0.095	−11.5	−11.2	25.6	25.1	49.3	−0.016
21 [a]	28.7	27.7	43.5	−0.152	−11.5	−11.4	26.0	24.5	49.6	−0.010
21 [b]	26.3	28.6	45.0	−0.116	−11.4	−11.4	24.1	27.0	48.9	−0.025
22	25.1	25.9	49.0	−0.23	−8.3	−11.7	25.1	25.8	49.1	−0.021
23	–	–	–	–	–	−8.2	–	–	–	–
24	–	–	–	–	–	−8.5	–	–	–	–
31	26.4	24.4	49.2	−0.018	−8.4	−8.6	25.9	24.3	49.8	−0.004
32	27.5	24.5	48.0	−0.046	−8.3	−8.4	25.1	26.8	48.1	−0.044
41	–	–	–	–	–	−8.5 [c]	–	–	–	–

[a] Mixture of cyanomethemoglobin C with unligated hemoglobin A_0.
[b] Mixture of cyanomethemoglobin A_0 with unligated hemoglobin C.
[c] Determined by analytical gel chromatography.

rapidly hybridizing species which are probably related to the methemoglobin content of the sample of deoxyhemoglobin A_0 (1–2%). When deoxyhemoglobin C, containing a greater amount of methemoglobin (2–4%), was incubated with cyanomethemoglobin A_0, the amount of rapidly hybridizing components was in the range of 4–8%. However, at equilibrium the concentration of $(\alpha^{CN}\beta^{CN})(\alpha^{deoxy}\beta_c^{deoxy})$ was found to be only slightly higher (1–2% higher) than that of $(\alpha^{CN}\beta_c^{CN})$ $(\alpha^{deoxy}\beta^{deoxy})$ (see table 1).

A plot of concentration vs. incubation time of the 'species 22' hybrid $(\alpha^{CN}\beta_c)$ $(\alpha\beta^{CN})$ is shown in fig. 3 along with those of the two parent species. The amount of hybrid found after only 4 min incubation was 37%, indicating that both the parent species hybridized rapidly. At equilibrium the distribution was (1 : 2 : 1).

A summary of the data on the equilibrium distribution of species obtained by hybridization of all combinations of symmetrical parent molecules is shown in table 1. The analysis of these distributions in terms of free energy values is also listed. The free energy deviations from additivity δG were calculated using eq. 3. To calculate ΔG_{AB} according to eq. 2, these values were combined with the assembly free energies for the parent species 01, 23, 24, and 41 presented by Smith and Ackers [2]. In the present experiments, the free energies of hybrid formation were found to differ by less than 0.2 kcal from the mean value of the assembly free energies of the parent species. This correspondence was obtained for the deoxy- and ligated conditions.

These findings of free energy additivity are consistent with the results reported by Smith and Ackers [2] for five of the six species studied here, but a discrepancy exists with regard to species 22. Smith and Ackers determined an apparent assembly free energy of -11.7 ± 0.2 kcal based on an analysis of the kinetic parameters which, by eq. 2, yields a value of 3.3 ± 0.3 kcal for δG. Via eq. 3 this leads to the prediction of vanishingly small fractions of parent molecules in the equilibrium mixture (i.e., $< 1\%$). Such a dramatic deviation from the essentially binomial distribution observed in the present study lies far beyond the expected error limits of both the kinetic method

used by Smith and Ackers and the equilibrium method used in this study.

We believe that the most likely source of this discrepancy is the presence of small amounts of unligated hemoglobins (species 01) in the initial reaction mixtures of species 23 and 24. This species, which is extremely difficult to eliminate completely, leads to the formation of small amounts of species 11 and 12 through combination of its dissociated dimers with those of species 24 and 23, respectively. Small amounts of species 11 and 12 have a much larger effect on the kinetic method, which relies solely on the detection of kinetic phases, than it does on the equilibrium experiments, which rely solely on 'amplitude' information. In the experiment of Smith and Ackers, a kinetic phase corresponding to -11.7 kcal was observed (probably due to small amounts of contaminating species 11 and 12) while any hybrid with an assembly free energy of -8.3 kcal (as found in this study) would not be detectable as a species distinctly different from the parents. Recent experiments in which extraordinary efforts have been made to eliminate species 01 in initial reaction mixtures of species 23 and 24 have failed to detect the kinetic phase corresponding to -11.7 kcal (Shea and Ackers, unpublished results).

Additional evidence for the effect of contaminating species 01 can be seen in the time course behavior shown in fig. 2 for mixtures containing initial samples of species 23 or 24. It is observed that whenever hybridization involves either of these species, i.e., the species which should hybridize rapidly, the amount of hybrid found after short incubation time is about 40%. This fraction is found to be approximately the same after 1–2 h. It reaches its final binomial value only after short incubation times is about 40%. This fraction is found to be approximately the same after 1–2 h. It reaches its final binomial value only after many hours of equilibration. While further studies may be necessary to delineate fully the exact sources of kinetic uncertainty in both techniques, the equilibrium results obtained with the present cryogenic experiments could not be explained if the assembly free energy of species 22 were as large as -11.7 kcal. We believe that the correct value, obtained by combining data from both the kinetic and equilibrium techniques (table 1), is very close to -8.3 kcal.

distinct values for the free energies of hemoglobin assembly as reported by Smith and Ackers [2]. The distribution of the three assembly free energies found in this study (table 1) also provides further support for the concept of a combinatorial switch as proposed by Smith and Ackers [2], i.e., the energetics of cooperative interaction depend not only on the number of ligands bound, but also on the specific configuration of ligated subunits. The simple two-state MWC model (the 'concerted' model), by contrast, requires that all tetrameric species with a given number of ligated sites should behave identically with respect to their cooperativity and subunit assembly properties [3,13].

Acknowledgements

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