Eur Biophys J (1988) 16:101–108

European Biophysics Journal

Distinct affinity and effector residues in the binding site for a regulatory ligand

The mitochondrial uncoupling protein as a model

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Received June 11, 1987/Accepted in revised form December 21, 1987

Abstract. A hypothesis concerning two distinct classes of amino acid residues in some regulatory binding sites is proposed. The "affinity residues" are those that are unable to transduce the ligand information signal but are responsible for overcoming the barrier for the attachment of a ligand to its binding site while the "effector residues" transfer the binding signal to the other functional part of the protein, which then undergoes a non-equilibrium energetic cycle induced by interaction with the ligand.

As an example, the purine nucleotide inhibition of H^+ transport through the uncoupling protein of brown adipose tissue mitochondria is discussed; there is a concentration range in which the nucleotide is bound but does not inhibit H^+ transport. This is interpreted in terms of inaccessibility of the effector residues inducing H^+ transport inhibition below a certain threshold concentration.

Key words: Regulatory binding site, affinity and effector residues, allostery, nucleotide-binding sites, uncoupling protein

Introduction

Ligand binding to proteins resulting in an effect on their function can be considered as a general biological phenomenon. Such processes comprise transfer of information (Daggett and Schuster 1984) from regulatory to catalytic sites of allosteric enzymes (Koshland and Neet 1968; Citri 1973; Fersht 1977), transfer of information via hormone receptors (Ariëns et al. 1979; Schuster and Levitzki 1980; Kenakin 1984, 1985; Dohlmann et al. 1987) and via chemically activated ion channels (Hille 1984; Maelicke 1986). The latter falls into the category of ligand-induced activation or inhibition of transport proteins (cf. Martonosi 1985).

Koshland's induced-fit theory represents a well established model of ligand-protein interaction emphasizing the flexibility of the ligand-binding site and its response to binding the ligand (Koshland and Neet 1968; Citri 1973). A classification of substrate or product interactions with the active site of an enzyme was carried out by Jencks (1975). In addition to the induced-fit mechanism he considered non-productive binding as a means of ensuring the specificity for a particular substrate. Recent knowledge of dynamic phenomena in proteins (Welch 1984, 1986; Karplus and McCammon 1983) completes the picture of ligand-protein interactions and has led to the development of a theory of allostery which does not consider large conformational changes as a necessary step but emphasizes specific modes of vibration or shifts in the mean of the probability distribution of conformational substates which conduct information across the protein structure (Cooper and Dryden 1984). Selection of conformations is also considered as a cause of intrinsic receptor efficacy (Kenakin 1985).

The attempts are focused now on finding the minimal structure of a proper ligand and the minimal architecture of the ligand-binding site. The question may arise whether free energy of ligand association with a protein (or the chemical potential) can depend on the extent of the reaction. In the case of association-dissociation of protein subunits it was suggested that a conformational drift is probably the cause of a continuous loss of the free energy of association accompanying an increasing degree of dissociation (Xu and Weber 1982; Weber 1986) and that the principle of detailed balance which does not allow such behaviour has a limited meaning in this context when a reaction is determined by the specific architecture and the dynamics of the protein reactive site (Weber 1986). It is also supported by Blumenfeld's (1976, 1981) "molecular machines" theory.

Here we have tested whether the individual amino acid residues in the protein active site can be assigned

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specific roles in the interaction with a regulatory ligand. This has led to a hypothesis that assumes two distinct classes of residues, the *affinity* and the *effector* ones, which could be involved in the binding sites for larger ligands.

The hypothesis is supported by data on purine nucleotide interaction with the uncoupling protein of brown adipose tissue mitochondria (cf. Kopecký et al. 1987). The uncoupling protein was used as an example exhibiting both ligand-induced activation of H^+ transport by fatty acids (Strieleman et al. 1985) and ligand-induced inhibition by purine nucleotides (Nicholls et al. 1974; Heaton and Nicholls 1977; Kopecký et al. 1984, 1987). The protein has been isolated (Lin and Klingenberg 1982; Klingenberg 1984), reconstituted (Strieleman et al. 1985; Klingenberg and Winkler 1985) and its amino acid sequence has been determined (Aquila et al. 1985). The striking disproportion between binding of GDP and its inhibition of H^+ transport (Kopecký et al. 1987) can be explained on the basis of the proposed hypothesis.

Affinity and effector residues in the binding site for a regulatory ligand

Phenomenological description

A ligand binding site of a protein consists of several amino acids, some of which interact with the ligand by strong attractive forces but cannot transfer the binding signal to the other part of the protein. As the free-energy barrier for a ligand to attach itself to the binding site is high (Rappaport 1976; Hill 1977) such residues are instrumental in overcoming the barrier and, consequently, determine the affinity of binding. Let them be referred to as the affinity residues and their whole as the affinity part of the binding site (Fig. 1).

On the other hand, residues that transfer the binding signal to the other part of the protein must form a part of the site to mediate the ligand-induced effect (Fig. 1). Let them be referred to as the effector residues and their whole as the effector part of the binding site. After interaction of the ligand with these residues the signal is transferred either by a conformational change or through specific vibrational modes (Cooper and Dryden 1984) which affect residues included in the structure of the channel, active site or the other part of the protein (functional residues). It is also possible that the effector residues coincide with the functional residues in some particular cases. Such residues were characterized as specificity residues (Koshland and Neet 1968). The existence of residues exhibiting the properties of both affinity and effector residues cannot be excluded.

Thermodynamic basis

If the interactions between the affinity and the effector residues are negligible one can derive the Gibbs free energies of interaction of the ligand L with the appropriate subsystem of protein macromolecules bearing the appropriate residues (P_a for the affinity and P_e for the effector part of the protein). Such arbitrary dissection of the protein can have an important structural basis as most receptor proteins (Dohlman et al. 1987) and also the uncoupling protein (Aquila et al. 1985) possess a loop structure with several spans across the membrane; as the loop parts are exposed to the external solution they interact with the ligand and it is no problem to envisage the affinity and effector residues to be localized on different loops.

Supposing that ligand interaction with the affinity residues occurs first we have

$$P_a + L \rightleftharpoons P_a L$$

and, followed by the interaction with the effector part,

$$P_a L + P_e \rightleftharpoons P L.$$

Differences in the Gibbs free energies during the interaction will be given by

$$\Delta G_a = E(P_a) + E(L) + E(P_a L) - kT \ln Q(P_a) \cdot Q(L)/Q(P_a L) \quad (1)$$

$$\Delta G_{e1} = E(P_{a1} L) + E(P_{e1}) + E(P_1 L) - kT \ln Q(P_{a1} L) \cdot Q(P_{e1})/Q(P_1 L), \quad (2)$$

where E's are the ground-state energies of the hypothetical binding at 0 K while Q's designate the corresponding partition functions (McQuarrie 1976). P_{a1}, P_{e1} and P_1 correspond to the state of the affinity and effector part of the protein and of the whole protein, respectively, at the moment of interaction with the effector residues. This state approaches the second state P_2, e.g., the state of affected regulation, $P_1 L \rightarrow P_2 L$. While the protein affinity part P_a does not change its structure, the protein part P_e which includes the functional residues is changed during this transition (the functional residues change their localization so that transport or an enzyme reaction is interrupted). Consequently, ligand interaction with the affinity residues should serve to stabilize the energy minimum and ΔG_a should not vary very much during the shift from state 1 to state 2, while in the case of the effector residues the energy minimum is reached only after transduction of the binding signal (Fig. 1).

Dynamic aspects of ligand binding and interaction

The canonical partition function of the ligand, $Q(L)$, and the protein subsystems $Q(P_a)$ and $Q(P_e)$ involve vibrational and conformational terms as the most

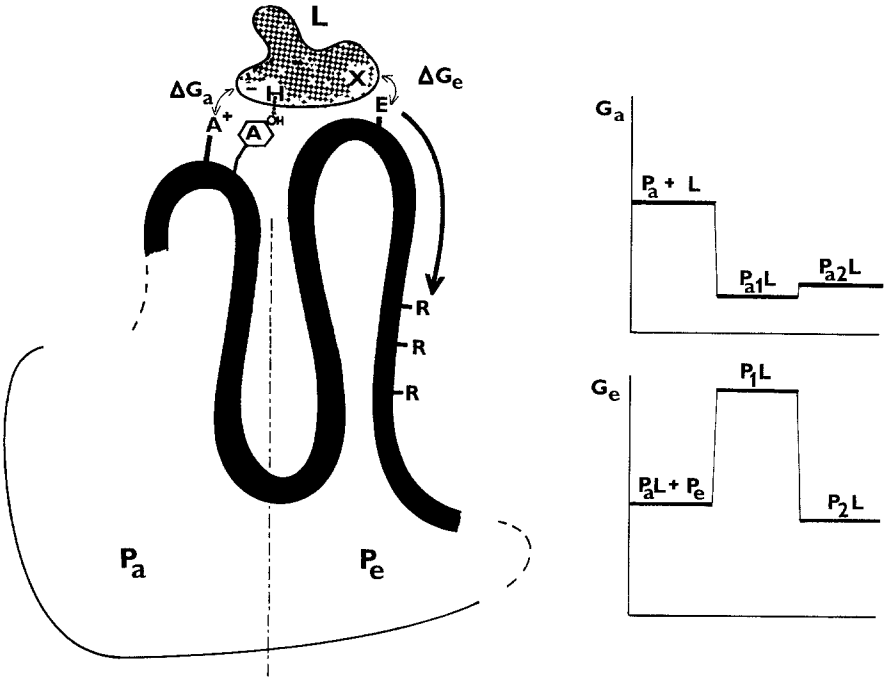

Fig. 1. Hypothetical binding site for the regulatory ligand composed of affinity amino acid residues (A) and effector residues (E) which transfer the binding signal to the functional residues (R) (for example, in the transport pathway). Three types of interactions between the ligand and protein are illustrated: electrostatic, hydrogen bond and van der Waals interactions. The protein tertiary structure was tentatively broken down into the affinity part P_a, bearing the affinity residues and not involved in the information transfer, and the effector part P_e which contains the effector residues (E) and a link transducing the binding signal to the functional residues (R).

The *right-hand part* shows the predicted changes in the Gibbs free energies of these parts of the protein (ΔG_a and ΔG_e) during binding and transfer of the effector part from state 1 (predominating in the absence of ligand) to state 2 (after effector residue interaction).

important parts (Cooper and Dryden 1984). After interaction with the ligand, concerted larger vibrations can be induced among vibrational modes or certain conformational substates can be stabilized (phenomenologically, a conformational change has taken place) (Cooper and Dryden 1984; Blumenfeld 1976, 1981). (To distinguish between the change of vibration and the relaxation into a new conformational substate, one must recall the definition of a conformational change as a change whereby a new set of weak interactions, hydrogen bonds, hydrophobic and van der Waals interactions, between atoms are established. Therefore, each conformational substate of a residue exhibits its own set of weak interactions with its surroundings whereas only the average positions in space are changed upon a change of vibration.) It seems reasonable to assume that not all residues in the binding site are such that ligand interaction with them induces the modes of vibration or conformational change that serve as information channels, but the effector residues do.

In contrast, the affinity residues participate in vibrational modes or conformational substates which are modulated after interaction with the ligand so that they cannot influence the other protein function.

When the interaction of the ligand with both types of residues occurs simultaneously no exceptional kinetics of regulation (inhibition, activation) can be observed. However, when the effector residues require for the interaction a time Δt_e longer than that required for the affinity residue interaction (Δt_a) one can expect an exceptional response because the effect cannot be switched on until $\Delta\tau > \Delta t_e$, where $\Delta\tau$ is the average time a ligand resides at the binding site.

It is usually considered that transitions between states occur instantaneously on the time scale of the associated kinetics (Hill 1977). Only the model of Weber (Xu and Weber 1982; Weber 1986) considers a "conformational drift" to occur when dissociating monomers cease to influence each other and assumes the establishment of reaction equilibrium in a shorter time than that required for conformational changes. Consequently, it predicts that the chemical potential of a dimer and, therefore, the free energy of the reaction can change according to the extent of the reaction. The principle of detailed balance is seemingly violated here but actually it is not relevant to protein interactions. Analogously, in the case of delayed effector residue interaction the substates $P_a L$ and $P_a P_{e1} L = P_1 L$ can occur within a longer time interval than $\Delta\tau$. While the

Δt_a is inversely proportional to the rate of ligand dissociation and independent of concentration, with increasing concentrations the frequency of collisions increases which can lead to superposition of the times Δt_a so that the total interaction time $\Delta\tau$ becomes longer than Δt_e. Thus the effect can only be induced above a threshold concentration c_t corresponding to the frequency when $\Delta\tau = \Delta t_e$. Consequently, the chemical potential of the ligand upon interaction with the effector residues changes with the extent of the reaction in the same way as such changes proceed during the conformational drift of monomer dissociation in Weber's (1986) model.

The cause of the delayed effector residue interaction can be seen in the different distribution of conformational/vibrational substates of the protein effector part P_e with regard to the affinity part P_a. Substates of the effector residues should also depend on the substates of the functional residues in the channel, in the active site or the other functional part of the protein, possibly in the manner shown by the energy diagram in Fig. 1. Discrepancies between binding and gating can thus arise from asynchronous interactions of the ligand with the affinity and the effector part of the regulatory binding site. Various magnitudes of the intrinsic efficacy exhibited by the ligands with an approximately equal binding constant (cf. Kenakin 1985) would be given by the different Δt_e values resulting from the different nature of the appropriate parts of ligand molecules. Such ligands would exhibit different values of the free energy of interaction $\Delta G_{e1} = G(P_1 L) - G(P_a L + P_e)$ (cf. Fig. 1) and they can even differ in their specific chemical potential dependences on the effect of interaction with the effector part. Simply speaking, they can differ in the mechanism of interaction.

Experimental distinction between affinity and effector residues

Existence of a latent concentration range in the effector response

According to the above considerations one can predict the existence of a latent concentration range ($c < c_t$) in which the ligand interacts with the affinity residues but produces no effect because of the delayed effector residue interaction. In this range binding would be observable and per cent saturation of the total amount of binding sites p could be increased up to threshold saturation p_t without eliciting an effect.

As an example let us take the inhibition of a transport pathway (channel) after binding a ligand. The fraction of closed channels n_{closed} will be zero when $p < p_t$. Assuming that above the threshold occupancy p_t the value of n_{closed} increases in proportion to increased occupancy and, taking into account that at $p = 1$ the value of n_{closed} must be unity, one can write

$$w = 1 - n_{\text{closed}} = 1/(1 - p_t) - p/(1 - p_t) \tag{3}$$

for $p > p_t$. Here w is the relative transport rate $v/v_{c=0}$. The value of w is unity if $p < p_t$. As p in general is given by the quasi-equilibrium constant K_d we have

$$p = (c/K_d)/(1 + c/K_d) \tag{4}$$

and one can substitute Eq. (4) into Eq. (3) to obtain

$$w = 1/(1 - p_t)(1 + c/K_d). \tag{5}$$

When no delayed response occurs ($\Delta t = 0$) so that $p_t = 0$, the concentration at which the transport is reduced by 50% (IC_{50}) will coincide with the value of K_d. If $p_t \neq 0$, the following relationship should hold:

$$K_d = (1 - p_t)/(1 + p_t) \cdot IC_{50} = \xi\, IC_{50}. \tag{6}$$

Thus, delayed interaction of the effector residues is indicated by the non-identity of the binding parameter K_d and the functional parameter IC_{50}. Such lack of correlation between the binding and the effect in the case of a protein bearing one ligand-binding site could reflect the characteristic difference between stronger interaction inherent in the affinity residues and a weaker, but functionally important, interaction of the effector residues. Disproportionality between the binding and its effect is well known from pharmacological studies (Kenakin 1984, 1985) and terms, such as "binding affinity" and "efficacy" could describe the properties of affinity and acceptor residues in single-binding-site receptors.

Chemical identification of the affinity and effector residues

A number of experiments can be proposed to identify residues related to affinity or effector response. In the ideal case when all residues in the regulatory binding site are identified, it will be necessary to evaluate their interaction energies at all possible stages (see Fig. 1) and to judge which single residue changes interaction energy within a functional cycle more and which changes it less. So far, this kind of approach has only been possible in the case of the active site of the tyrosyl-tRNA synthetase (Fersht et al. 1986) where protein engineering was employed.

With unidentified residues one can use classical approaches, chemical modifications or studies with a series of ligand analogues. Since the interaction of the ligand with the effector site can only induce the regulatory effect, any chemical modification or mutation of

the effector residues should prevent it. In contrast, a chemical modification or mutation of the affinity residues should not abolish the effect response to the regulatory ligand totally but should decrease the affinity of the binding site. Studies with ligand analogues could define ligand atoms or groups essential for the interaction with the corresponding amino acid residues in the binding site. The groups responsible for the highest binding affinity may interact exclusively with the affinity residues.

On the other hand, groups or atoms found to be essential for the effect should interact with the effector residues. For a review in the case of ATP-binding sites of the H-ATPase and the ADP/ATP carrier as examples of such studies see Vignais and Lunardi (1985).

Purine-nucleotide-binding site of the uncoupling protein as the paradigm

We have recently shown (Kopecký et al. 1987) a biphasic dependence of GDP inhibition of H^+ transport via the uncoupling protein on the occupancy of the detectable ^{3}H-GDP-binding sites in intact brown adipose tissue mitochondria. Up to 50% occupancy, no inhibition was observed and, between 50% to 100% occupancy, the inhibition increased linearly with the occupancy. Chemical modification by *p*-diazobenzenesulfonate (DABS) caused a shift of the break of the curve to 95% occupancy but the inhibition progressed linearly with the occupancy between 95% and 100%. Chloride transport which is regulated by the same binding site as the H^+ transport but which proceeds through an independent transport pathway (Kopecký et al. 1984) did not exhibit a latent concentration range. This explains its higher sensitivity to purine nucleotides. However, a biphasicity was induced by DABS (Kopecký et al. 1987).

A detailed analysis of GDP inhibition of H^+ and Cl^- transport through the uncoupling protein at intermediate pH (7.05) is shown in Fig. 2a. While reciprocal relative rates of Cl^- transport are linearly proportional to increased GDP concentration, a clearly biphasic character of the Dixon plot for the inhibition of H^+ transport is observed (linear regression by two straight lines yielded an r^2 of 0.98 while parabolic fitting gave an r^2 of 0.24). Up to 20 μM GDP no significant decrease in H^+ transport activity is observed, hence this region can be defined as the latent concentration range. The data are compared with those published elsewhere (Kopecký et al. 1987), measured at lower pH (6.8) where inhibition is more intense, as shown by a steeper Dixon plot in the controls (Fig. 2b). The inhibition exhibits a highly extended latent concentration range in mitochondria modified by DABS (Fig. 2b); up to 300 μM GDP when 75 nmol DABS/mg protein was used.

To explain how the uncoupling protein senses the external purine nucleotide concentration and how it is able, only above a threshold concentration, to transfer the binding signal to the gate of the H^+ channel the above hypothesis can be invoked. Table 1 summarizes the calculations according to the threshold occupancy model derived in the preceding section. A fine agreement between the proportionality coefficients calculated according to Eq. (6) and the measured IC_{50}/K_d ratios was reached. As it is well established that the uncoupling protein possesses a single GDP-binding site per dimer (Lin and Klingenberg 1982) and as our data also fit only a single-site model (Kopecký et al. 1987) one can exclude any cooperativity effects as the cause of the described phenomena (cf. Mazat 1977). This is supported by the Hill coefficients which were significantly different from one only within the inhibitory phase ($c > c_t$) in DABS-modified mitochondria (Table 2).

All the results including chemical modifications support the following explanation built on the above hypothesis. The affinity residues of the uncoupling protein nucleotide-binding site can interact with and bind purine nucleotides in the low (latent) concentration range, when no inhibition is detected. Overcoming the threshold concentration c_t allows the ligand to interact also with the effector residues and to develop an inhibitory response. In addition to the mechanism of superposition of interaction times Δt_a proposed above this could also be effected by a conformational change of the nucleotide binding site structure which makes the effector residues accessible.

Diazobenzenesulfonate probably modifies the affinity residues by reducing the affinity of binding (Kopecký et al. 1987) and prevents the interaction of the nucleotide with the effector part of the nucleotide binding site, either sterically or by stabilization of the

Table 1. Proportionality between K_d for GDP binding and IC_{50} for GDP inhibition of H^+ transport in the case of control and brown adipose tissue mitochondria modified by diazobenzenesulfonate (DABS). Coefficients ζ were calculated according to the threshold occupancy model, $\zeta = (1 - p_t)/(1 + p_t)$

Conditions	Measured[a]			Calculated	
	IC_{50}	K_d	p_t	ζ	K_d/IC_{50}
Controls, pH 6.8	7.4	2.7	0.470	0.361	0.365
25 nmol DABS per mg protein	38.0	7.9	0.660	0.205	0.208
75 nmol DABS per mg protein	560	12.9	0.950	0.026	0.023

[a] Data taken from Kopecký et al. (1987)

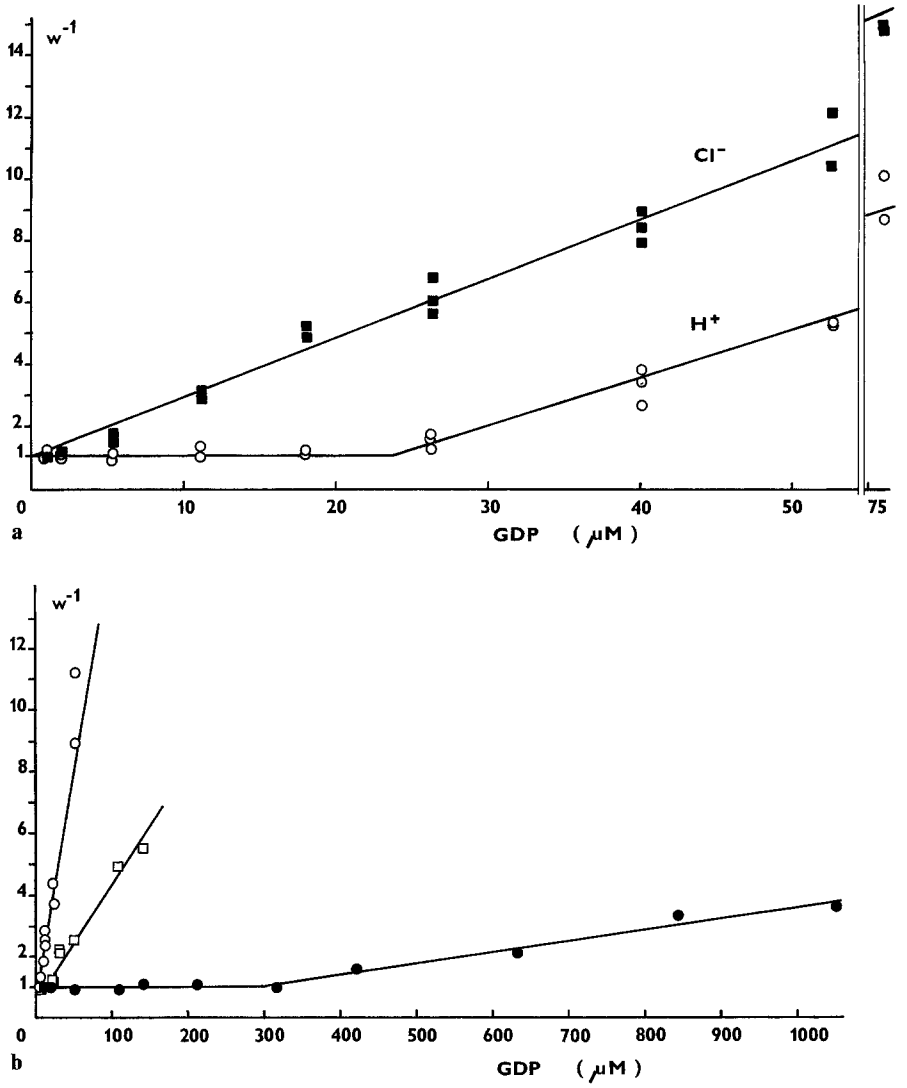

Fig. 2a. Dixon plots of GDP inhibition of H^+ and Cl^- transport through the uncoupling protein at pH 7.05 in intact brown adipose tissue mitochondria. o, H^+ transport – valinomycin-induced H^+ extrusion in KCl; ■, Cl^- transport – simultaneously recorded swelling. Relative transport rates $w = v_{GDP}/v_{GDP=0}$ are plotted as reciprocal values against GDP concentration. The data were fitted by linear regression using the least-squares method ($r^2 = 0.98$ in both cases). **b** Dixon plots of GDP inhibition of valinomycin-induced H^+ extrusion in KCl in brown adipose tissue mitochondria modified by *p*-diazobenzenesulfonate (DABS) at pH 6.8. (Data from Kopecký et al. 1987) o, Control; □, 25 nmol DABS/mg protein; ●, 75 nmol DABS/mg protein. Each point represents the mean of at least four independent measurements. Linear regression yielded fits with r^2 0.88, 0.97 and 0.96, respectively.

Table 2. Hill coefficients for GDP and ATP inhibition of H^+ and Cl^- transport through the uncoupling protein in intact and diazobenzenesulfonate-modified brown adipose tissue mitochondria. Each experiment represents at least 10 titration points in duplicate or quadruplicate. Hill expressions $-\log(1/w - 1)$ were calculated for each point and plotted against log [GDP]. The dependences were linearized by linear regression using the least-squares method. Goodness of fit was not below 0.85. The Hill coefficients were then taken as slopes of these straight lines

Transport	GDP inhibition							ATP inhibition		Inhibition by albumin
	Exp. I[a]	II[b]	III[b]	IV[b]	V	75 nmol DABS[b] [mg protein]$^{-1}$	25 nmol DABS[b] [mg protein]$^{-1}$	Exp. I[a]	II	
H^+ extrusion	0.86	1.28	1.18	1.07	1.02	1.77	1.67	1.06	1.03	0.81
Cl^- uptake (swelling)	1.19	1.5	0.80	1.24	0.71	0.70	0.96	0.60	0.89	–

[a] Data taken from Kopecký et al. (1984)
[b] Titrations of three different mitochondrial preparations II, III, IV. The average data were presented in Kopecký et al. (1987)

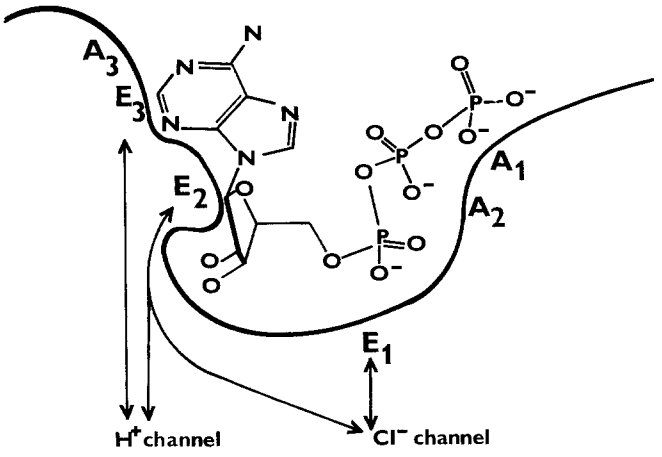

Fig. 3. A hypothetical view of the location of affinity and effector residues in the purine-nucleotide-binding site of the uncoupling protein of brown adipose tissue mitochondria. As purine nucleoside di- and triphosphates exhibit a much higher affinity for the ligand, the two affinity residues were tentatively selected to interact with γ- and β-phosphate groups. The possible existence of further affinity residues attracting the adenine ring is shown by residue A_3. As the minimal structure of an effective compound should be composed of adenine (or guanine or inosine) ring, ribose and α-phosphate, three distinct effector residues were placed so as to interact with those ligand parts. One effector residue must interact with a region close to ribose as a nucleotide analogue with dimethylaminonaphthoyl group attached to ribose also binds but does not inhibit (Klingenberg 1986). The differential gating of the H^+ and Cl^- channels can be effected by the effector residue exclusively transducing the binding signal only to the H^+ channel which is accessible only above the threshold concentration c_t (residue E_3). To complete the model it is necessary to assume that one of the affinity residues is an ionizable group causing the affinity to decrease with increasing pH (cf. Klingenberg and Winkler 1985).

state of the latent concentration range $c < c_t$, or by abolishing the conformational transition.

Because purine nucleoside monophosphates do not inhibit so strongly (Nicholls et al. 1974; Heaton and Nicholls 1977) and bind to the uncoupling protein with a lower affinity (Klingenberg 1984; Lin and Klingenberg 1982), while pyridine nucleotides do not even bind to the uncoupling protein, one can deduce that the β- or the γ-phosphate group interacts mainly with the affinity part of the nucleotide binding site and some atoms of adenosine, guanosine or inosine interact with the effector residues (Fig. 3).

The uncoupling protein should possess at least two effector residues – one for gating the Cl^- channel and the second for gating the H^+ channel which must be accessible only after overcoming the threshold concentration c_t, while the former effector residues should interact with nucleotides just at the moment when the affinity residues attract them. This mechanism most probably ensures the differential gating of two transport pathways within one dimeric protein from the common regulatory binding site.

Conclusion

The induced-fit theory (Koshland and Neet 1968) considered the receptor specificity to be predetermined by the structure and the functional specificity to be determined by the response of the receptor site to the structure of the ligand (Citri 1973). We would like to emphasize that the response at the molecular level is mediated by particular amino acid residues (the effector residues) which occur in a non-equilibrium state immediately after interaction with the ligand (cf. Blumenfeld 1976, 1981) and which, by conformational or vibrational relaxation, communicate with the other part of the protein that becomes regulated. Not every residue in the binding site can be equipped for such a complex function. Among the residues there should be such as are responsible for the attraction of the ligand to the site – the affinity residues which are important for the maximum efficiency in binding the ligand.

Acknowledgements. The authors thank Dr. K. Janáček of the Institute of Microbiology, Czechoslovak Academy of Sciences, and Dr. I. Dvořák of the Institute of Psychiatry for stimulating discussions and critical reading of the manuscript.

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