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COMMENTARY

Temperature-Nearly-Independent Binding Constant in Several Biochemical Systems

THE UNDERLYING ENTROPY-DRIVEN BINDING MECHANISM AND ITS PRACTICAL SIGNIFICANCE

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ABSTRACT. Arguments are presented in this commentary to show that the model of temperature-nearly-independent binding that we proposed to rationalize the binding characteristics of β-adrenergic antagonists (Miklavc *et al.*, *Biochem Pharmacol* **40:** 663–669, 1990) in fact provides a consistent interpretation of the temperature-nearly-independent binding constant in all other systems that have been reported in the literature: in the binding of coenzyme NADH to horse liver alcohol dehydrogenase and to octopine dehydrogenase and in the binding of an inhibitor to acetylcholinesterase. No such consistent interpretation has been given thus far for any of these systems. It is characteristic of them that the binding takes place in a hydrophobic, sterically constrained environment. One can assume, therefore, that the underlying entropy-driven binding mechanism would reflect the existence and the properties of the steric bottleneck surrounding the binding pocket. We also explain why the temperature effects characteristic of hydrophobic interactions are not found experimentally in these systems, whereas in other, sometimes even structurally similar, systems such temperature effects are clearly present. Further work is necessary to establish more firmly the key features of the temperature-nearly-independent binding mechanism that has been disclosed through our analysis. The binding mechanism in question not only appears in important biochemical systems, but also has the interesting property of being relatively unaffected by smaller structural changes. BIOCHEM PHARMACOL 51;6:723–729, 1996.

KEY WORDS. adrenergic β-receptor blockers; NADH dehydrogenase; acetylcholinesterase; cholinesterase inhibitors; binding sites; binding constant; temperature-nearly-independent

A striking aspect of data obtained in binding experiments is that in some systems there is a pronounced temperature dependence of enthalpy and entropy changes, ΔH^0 and ΔS^0, upon binding, whereas in other, sometimes structurally apparently quite similar, systems ΔH^0 and ΔS^0 are nearly temperature independent. Using a different formulation, it is not understood why in some systems the van't Hoff plots show pronounced curvatures, whereas in other, often apparently not so different, systems these plots are nearly straight. A further problem is how to understand the binding if the van't Hoff plot is straight and almost horizontal. In such systems, ΔH^0 is approximately 0 and the binding constant is nearly temperature independent. Four important examples of such binding that can be found in the literature are critically reviewed: binding of antagonists to β-adrenergic receptors, binding of coenzyme NADH to horse LADH† and to ODH, and binding of an inhibitor *o*ClB-BQ to AChE. Temperature-independent binding is driven by a positive change of entropy. It is argued that the hydrophobic mechanism, often assumed to be at the origin of this entropy increase, can be excluded with great certainty in the above cases. Experiments also show that entropy-driven binding can occur without any large-scale conformational changes sometimes assumed to cause the entropy increase. We present arguments to show that the same mechanism of entropy-driven binding provides a consistent interpretation in the four cases mentioned. On the basis of the same theory, it is possible to understand why the van't Hoff plots are sometimes curved and sometimes straight, even for systems that appear to be similar in structure. We will also point out the practical significance of the binding mechanism in question.

EXPERIMENTAL FINDINGS

Efforts to understand a particular ligand–receptor complex are often concentrated on NMR and X-ray structural studies that may produce rather impressive structural pictures of the complex and suggest the formation of specific molecular bonds. Despite the obvious value of such structural models, their in-

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† *Abbreviations:* LADH, liver alcohol dehydrogenase; ODH, octopine dehydrogenase; YADH, yeast alcohol dehydrogenase; AChE, acetylcholinesterase; and *o*ClB-BQ, 2,5-bis[[3-[diethyl(*o*-chlorobenzyl)ammonio]propyl]amino]benzoquinone.

formational content may be lacking in several important respects. Specifically, the structural picture may not provide insight into the origin of the binding affinity and specificity of the ligand. In addition, it may not give us information about the stability of the complex or about the nature of the forces that drive its formation. Often one is inclined to simplify the analysis by relying solely upon the properties of the interaction energy. However, in some cases, the binding constant may be largely controlled by entropic effects. Structure–activity studies that ignore these entropic effects may be inadequate. Therefore, determination of the complete thermodynamic binding profile can be of great assistance in the study of the binding mechanism. The difficulty here is that the interpretation of thermodynamic binding data in complex biological systems may be far from straightforward. Understanding the entropy-driven binding mechanism leading to the temperature-independent binding constant certainly is among the most intriguing unsolved problems in this field. Although it can be reasonably expected that the mechanism in question would, in some cases, occur in combination with other, better understood and more common binding mechanisms, the four systems selected in the present study are the most clear-cut examples of the temperature-nearly-independent entropic binding that we have been able to find reported in the literature thus far.

Weiland *et al.* [1] was the first to report that there is a fundamental difference between the binding of agonists and antagonists to the β-adrenergic receptor in turkey erythrocytes. Antagonist binding was found to be characterized by a small, positive or negative enthalpy change (ΔH^0) and a large increase of entropy (ΔS^0). The binding of agonists, on the other hand, was associated with a large decrease in enthalpy and a highly unfavourable decrease in entropy (Fig. 1). Other researchers later reported similar observations with β-adrenergic receptors in different systems. A large part of the rather extensive literature is quoted and briefly reviewed in Ref. 2. These conclusions were based on analysis of the temperature dependence of the dissociation constant K_D.

$$\ln K_D = \Delta H^0/RT - \Delta S^0/R \quad (1)$$

In the case of β-agonists and -antagonists, the van't Hoff plots ($\ln K_D$ vs $1/T$) are essentially linear, meaning that ΔH^0 and ΔS^0 are nearly temperature independent (in the range 273–310°K). Since $|\Delta H^0| \sim 0$ for β-antagonists, the corresponding K_D depends only weakly on temperature (Fig. 2). The K_D of β-agonists, on the other hand, shows pronounced temperature dependence, due to large ΔH^0 values. It should be pointed out that in the binding of, for example, dopamine agonists and antagonists, van't Hoff plots that are strongly curved are encountered [3], showing that ΔH^0 and ΔS^0 are temperature dependent in this case (Fig. 1). A similar strong temperature dependence of ΔH^0 and ΔS^0 is observed when ligands form a complex with host molecules in water (see, for example, Ref. 4 and the references quoted therein) or in the solvation of noble gases or hydrocarbons [5, 6]. This dependence can thus be attributed to the effect of the change of the state of water on binding, i.e. to the hydrophobic effects. To illustrate, the temperature dependence of ΔH^0 and $T\Delta S^0$ for the solvation of liquid neopentane in water is shown in Fig. 3.

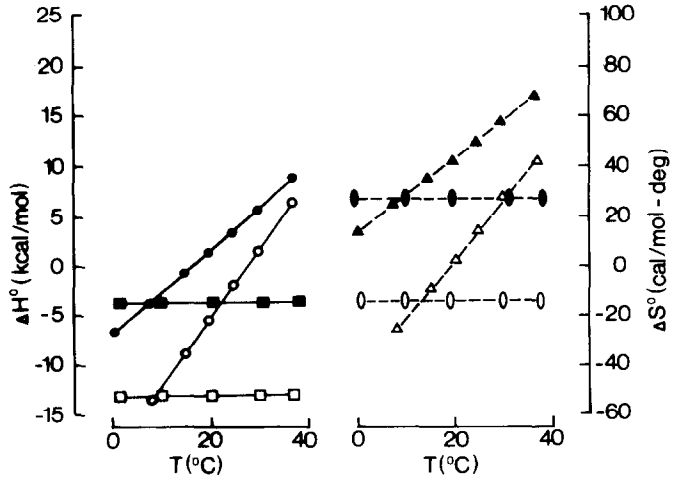

FIG. 1. Temperature dependence of the enthalpy and entropy changes, ΔH^0 and ΔS^0, in the binding of the dopamine antagonist haloperidol (●, ▲) and dopamine (○, △) (low affinity state) to the D_2-receptor [3], and in the binding of the agonist isoproterenol (□, ○) and the antagonist propranolol (■, ●) to the β-adrenergic receptor [1].

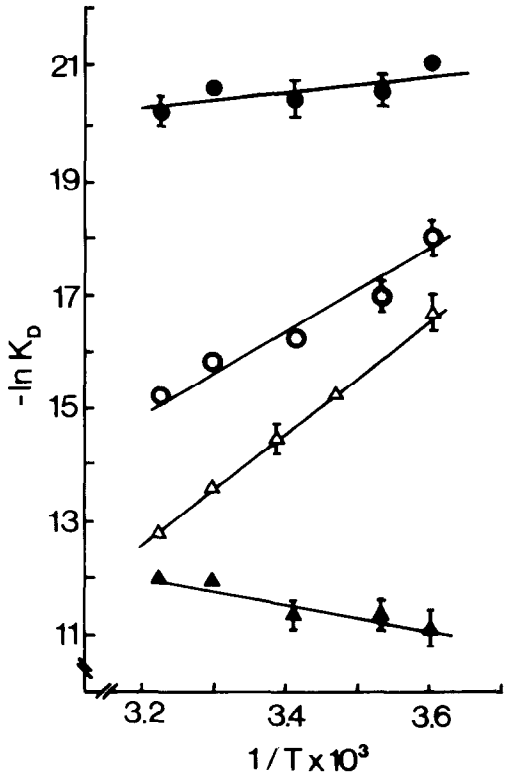

FIG. 2. van't Hoff plots of the dependence of K_D on temperature for the binding of several β-receptor ligands: β-antagonists (—)-propranolol (●) and practolol (▲) and β-agonists (—)-isoproterenol (○) and norepinephrine (△) [1]. Each point represents the mean ± SEM of 3–10 determinations done in duplicate.

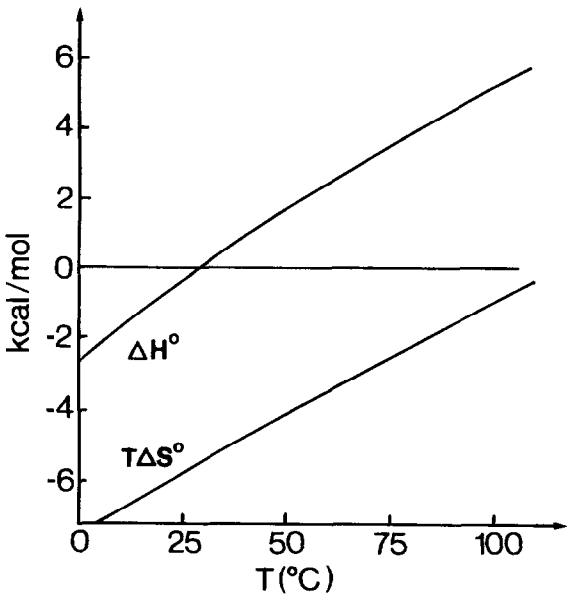

FIG. 3. Temperature dependence of ΔH^0 and $T\Delta S^0$ in the solvation of liquid neopentane in water [6].

We may thus conclude that the change of the state of water is reflected in the K_D of dopamine ligands, but it does not show up in the K_D of β-agonists and -antagonists. This conclusion is strongly corroborated by the fact that the K_D of the β-antagonist does not correlate with the experimentally determined lipophilicity indices [7]. Thus, the large entropy increase observed here cannot be of hydrophobic origin. The model that we will present below offers a possible explanation of this rather provocative conclusion.

Temperature dependence of the dissociation constant of the complexes of ODH with the coenzymes NADH, NAD and L-Arg + NADH in the temperature range 2.5–40° was studied by Luisi *et al.* [8], using fluorescence measurements. In all three cases, the dissociation constant was largely temperature independent (Fig. 4), indicating that ΔH^0 was approximately 0 and that the binding was thus totally entropy-driven (Table 1). It was pointed out in the original work [8] that such a mechanism may be Nature's trick for providing temperature regulation of biologically important functions in certain systems.

The thermodynamic parameters for the binding of NADH to some selected dehydrogenases have also been measured calorimetrically at $T = 25°$ [10]. Although the free energy changes (ΔG^0) in binding were of a similar magnitude, this similarity was rather superficial since the ΔS^0 and ΔH^0 components of ΔG^0 were found to differ widely from case to case. Binding of NADH to YADH, for example, is characterized by $\Delta G^0 = -6.3$ kcal/mol, $\Delta H^0 = -9.5$ kcal/mol, and $\Delta S^0 = -10.7$ cal/kmol, whereas $\Delta G^0 = -8.8$ kcal/mol, $\Delta H^0 = 0$ kcal/mol, and $\Delta S^0 = 29.5$ cal/kmol were found in the case of NADH binding to horse LADH. It has been pointed out [10] that the large differences in ΔS^0 values between the LADH–coenzyme complex and the complexes formed with the other enzymes cannot be accounted for in terms of differences, if any, in solvation and desolvation at the binding site, since in all cases the binding involved the transfer of NADH from the aqueous

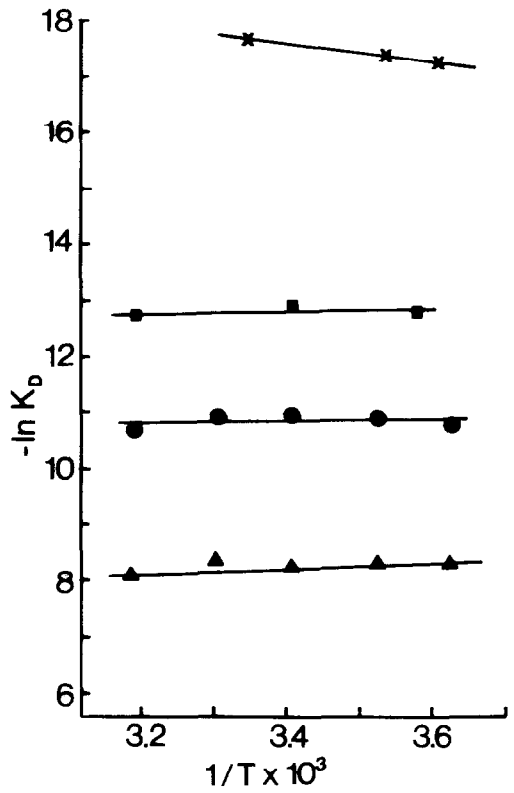

FIG. 4. van't Hoff plots of the dependence of K_D on temperature for: ODH + NAD (▲), ODH + NADH (●), and ODH · L-Arg + NADH (■) [8]. (x) are the corresponding results for oClB-BQ binding to AChE [9].

phase to the supposedly similar coenzyme binding site. The large positive entropy change (ΔS^0) was attributed, therefore, to a supposed conformational change that may occur in LADH but not in YADH. In crystal structure determination of LADH–H_2NADH complex [11], however, only local structural changes in the orthorhombic LADH were observed upon binding of the coenzyme analogue H_2NADH (Fig. 5). In contrast, the combined effect of the presence of H_2NADH and the substrate triggers the gross conformational change of the protein, involving the motion of the catalytic domains [11]. We may conclude, therefore, that in the cases in question ΔH^0 and ΔS^0 reflect primarily local interactions. The binding site of the coenzyme is located in a deep active site cleft that is largely hydrophobic. X-ray analysis revealed the positions of several water molecules bound in the cleft. A comparison between the

TABLE 1. Thermodynamic parameters of binding of coenzyme to ODH at 300°K

System	ΔG° * (kcal/mol)	ΔH° (kcal/mol)	ΔS° * (cal/mol · deg)
ODH + NADH	−6.4	−0.1	+21
ODH · L-Arg + NADH	−7.7	0.0	+25.5
ODH + NAD	−4.9	−0.1	+16

* Data from ref. [8].

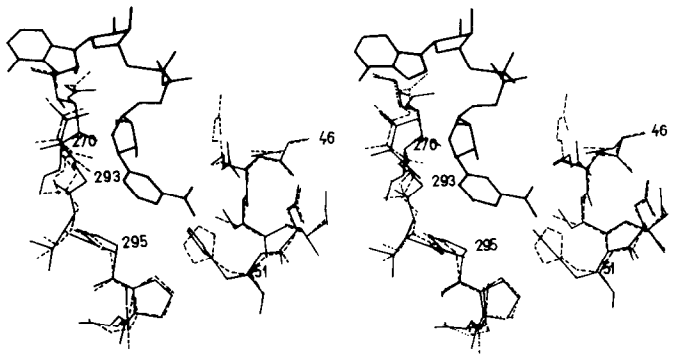

FIG. 5. Position of the H_2NADH molecule within the cleft between domains in the orthorhombic LADH. Thick, solid lines-the coenzyme analogue; thin, solid lines-unliganded enzyme. The local structural changes that occur upon H_2NADH binding are indicated by the dashed lines Reprinted with permission from *Biochemistry* 21: 4895–4908, 1982. Copyright (1982) American Chemical Society. [Ref. 11].

LADH–H_2NADH complex and the native enzyme, however, showed no great differences in the water-binding pattern of the cleft before and after the binding [11].

The most remarkable feature of the atomic structure of AChE is a deep and narrow gorge ("active site gorge"), about 20 Å long, that penetrates halfway into the enzyme and widens out close to its base [12]. It contains the AChE catalytic triad. The crystal structure of AChE also clearly reveals that the choline-binding anionic subsite is misnamed, as it contains, at most, one formal negative charge. Instead, the quaternary moiety of choline appears to bind chiefly through interactions with the π electrons in the aromatic residues [12]. The depth of the active site gorge, as well as the high aromatic content of the walls and floor of the gorge, may help explain the results of biochemical studies that disclosed a variety of hydrophobic and anionic binding sites distinct from, or overlapping, the active site. It has been pointed out [12] that, because of the depth of the gorge and its extensive aromatic surface, there must be many different ways and places for substrate, agonists and inhibitors to bind to AChE.

To the best of our knowledge, only the temperature dependence of K_D for the AChE inhibitor *o*ClB-BQ has been reported in the literature [9]. These results, displayed in Fig. 4, show a striking similarity with the other cases discussed above: the binding is entropy-driven, ΔH^0 = +2.64 kcal/mol, ΔS^0 = +44.0 cal/kmol, with ΔH^0 and ΔS^0 being nearly temperature independent. Thus, again K_D does not reflect any contribution of the hydrophobic effects. There is also a clear similarity with the LADH–coenzyme system in that the binding takes place in a sterically strongly constrained regime. As we shall argue below, this seems to be the key characteristic feature of all the systems with a temperature-nearly-independent binding constant presently known to us.

MECHANISM UNDERLYING THE TEMPERATURE-NEARLY-INDEPENDENT BINDING

We shall now present arguments to show that the mechanism of entropy-driven binding proposed in our earlier work [2] to explain the binding of β-antagonists in fact provides a consistent interpretation of data for all other cases of temperature-nearly-independent binding. In subsequent publications [13, 14], Searle and Williams discussed the problem of entropy changes in molecular association in considerable detail. They stress [13] that the correct interpretation must consider the entropy changes associated with transferring the ligand from solution to its final bound state. This is fundamentally correct as long as all parts of the system are in true thermodynamic equilibrium, which is a rather ideal situation [15]. If the thermodynamic quantities are determined from binding constant measurements, one *cannot know a priori which parts of the phase space are in thermodynamic equilibrium during the measurement process*. Thus, the outcome of the measurement may even depend on the measurement technique. A very striking example are the specific heat measurements in molecular gases [15]. As already discussed in the previous section, the pronounced temperature dependence of ΔH^0 and ΔS^0 could not be compensated throughout the whole temperature interval studied (0–40°) since no other mechanism with comparable temperature dependence is known. For this reason, and also for other reasons previously discussed, we are led to the conclusion that the binding constant measurements are decoupled from the solvation/desolvation effects in the cases of the temperature-nearly-independent binding. In fact, the same should hold true for all the systems characterized by linear van't Hoff plots. We shall be returning to this rather provocative conclusion below, showing that there may be a simple explanation, and giving experimental evidence in support of it.

It should also be mentioned here that often a conformational change is assumed as the origin of entropy increase in entropy-driven binding. The example of the H_2NADH + LADH complex, however, shows clearly [11] that in such binding processes only local structural changes may occur. Moreover, loose binding ($\Delta H^0 \sim 0$) of, say, β-antagonists could not be expected to induce substantial conformational changes: for the type of changes that can be expected in this case the observed ΔS^0 is too large.

In an attempt to find an explanation of the binding constant of β-adrenergic ligands that would be consistent with all the experimental facts, in particular with the observed temperature dependence, we suggested [2] that the binding data may, in this case, reflect a local equilibrium between the ligands in an enlarged binding cavity and those in the cavity's immediate hydrophobic surrounding where the ligands may be sterically severely constrained (Fig. 6). Upon transition from the surroundings into the cavity, β-antagonists do not form a tight bond ($\Delta H^0 \sim 0$) and thus may experience an increase of the rotational phase space leading to an increase (ΔS_r) of the rotational entropy that may outweigh the corresponding loss (ΔS_{tr}) of the translational entropy. The total entropy change (ΔS_{antag}) in binding of antagonists would then be

$$\Delta S_{antag} = \Delta S_{tr} + \Delta S_r > 0 \qquad (2)$$

in accord with the experiments. The β-agonist, on the other hand, may form a tight bond in the cavity (ΔH^0 large, nega-

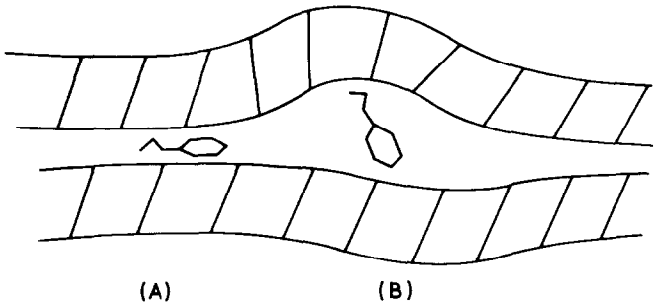

FIG. 6. Proposed model for binding with $\Delta S^0 > 0$. The ligand in the tight hydrophobic environment (A) is supposed to be in thermodynamic equilibrium with the loose (ΔH^0 small) bound states in the binding pocket (B) where the available phase space is larger than in (A), due mainly to less restrained rotational motion.

tive), which means that the corresponding changes (ΔS_{tr} and ΔS_r) would both be negative, leading to $\Delta S_{ag} < 0$ for the agonist. As pointed out in Ref. 2, this mechanism depends only weakly on the temperature (as ln T), which is also in accord with the data.

Soon after the model was proposed [2], the results of the fluorescence experiments on the binding of the antagonist carazolol to the β-adrenergic receptor were published [16, 17], which support the model in two crucial aspects. They provide direct physical evidence that the entire ligand binding site of the β-adrenergic receptor is buried deep within the transmembrane hydrophobic core of the receptor protein, with the carbazole group of carazolol bound more than 11 Å beneath the membrane surface (Fig. 7). These conclusions are consistent with the previous findings of the point mutation experiments [18, 19]. Furthermore, the polarization and the lifetime of the carazolol fluorescence are consistent with the proposed model of β-antagonist binding, as they indicate a fairly rigid environment of the ligand at the binding site and a rather weak interaction, which is comparable to that in the solvent. It now appears to be a view generally held [20] that, at least in the case of small amine ligands, the membrane-spanning helices form a pocket into which the ligand "fits."

A difficult question that remains to be discussed is why solvation/desolvation effects seem to be decoupled from the binding constant measurements of β-adrenergic ligands, but are apparently quite pronounced in the case of dopaminergic ligands. Recent investigations [21] based on molecular modeling and computational chemistry suggest that dopamine binds to the D_2 receptor, with its hydroxyl groups interacting with the extracellular disulfide bond formed between the TM3 and TM4 transmembrane domains (Fig. 8). These modeling data are also supported by recent biochemical findings [22]. Thus, dopamine seems to bind in the surface region of the membrane; its binding site may be at least partially in direct contact with the bulk water. The binding equilibrium, therefore, should reflect *direct* transition from and to the bulk water, which means that ΔH^0 and ΔS^0 should show a pronounced temperature dependence, which is, in fact, observed. Additional evidence in support of this view is given in Refs. 23 and

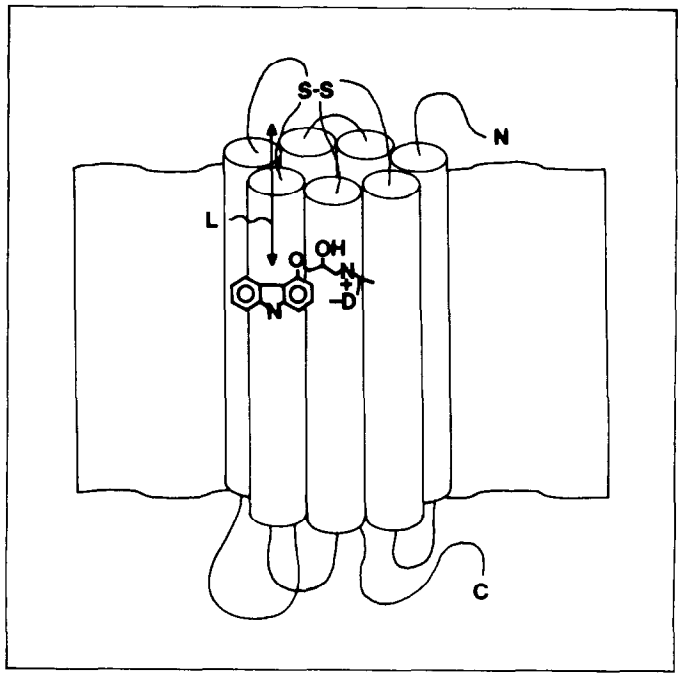

FIG. 7. Location of the antagonist carazolol in the binding site of the β-the adrenergic receptor proposed on the basis of the measurements of the fluorescence energy transfer [16]. Distance (L) of the carbazole group of carazolol from the membrane surface is greater than 11 Å. The seven putative transmembrane helices are shown as cylinders.

24, where it was found that the thermodynamic quantities ΔH^0 and ΔS^0 for agonist and antagonist binding to the dopamine D_2 receptor appear to be related to drug lipophilicity. No such correlations have been found in the case of β-adrenergic ligands [7]. It was also concluded that in this system ΔS^0 and ΔH^0 do not distinguish between the interaction of agonists and antagonists, in sharp contrast to the β-adrenergic system. In this respect, the thermodynamic studies [3, 23, 24] seem to agree, but they differ in the extent and details of the temperature dependence found. This may be due, in part, to the different biological systems being examined in these studies.

The model proposed is also consistent with the experimental findings in other systems with a temperature-nearly-independent binding constant, which have been reported in the literature. Most important, in all such systems the binding site is located in a narrow channel or cleft with largely hydrophobic walls, so that bulk water is not present there. Single water molecules that have been identified, for example in the X-ray structure of LADH [11], do not lead to hydrophobic effects. Moreover, in the particular case mentioned, they have been found to be largely unaffected by the binding. In all these systems, therefore, the ligand binding takes place in a sterically severely hindered regime. What remains to be done, however, is to establish directly by molecular modeling or various spectroscopic techniques, the existence of the steric bottleneck surrounding the binding site. Another important problem is how to explain that the solvation/desolvation effects apparently are decoupled from the binding constant measurements in such systems. The most direct measurements seem to be the

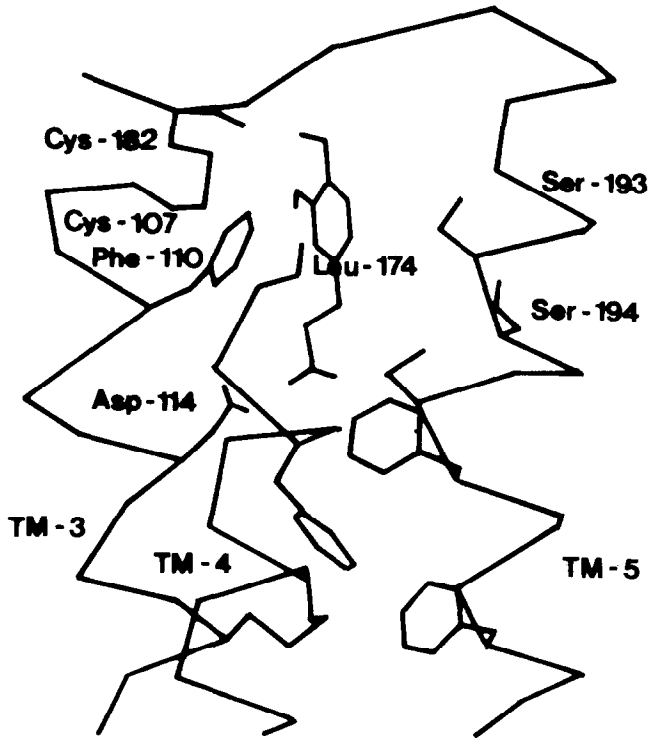

FIG. 8. Molecular model of the dopamine binding site that corresponds to the most stable complex found in recent computational studies [21]. In this complex, the hydroxyl groups of dopamine interact with the extracellular disulfide bond between Cys^{107} and Cys^{182}. These modeling data could also explain recent biochemical findings [22].

enzyme–coenzyme dissociation constant measurements in the case of ODH (see Ref. 8 and the references cited therein). They were based on monitoring the protein fluorescence quenching upon coenzyme binding and/or, in the case of NADH, by monitoring the enhancement of the coenzyme fluorescence. The dissociation constants have been determined from the ratio $\Delta F/\Delta F_{max}$. ΔF_{max}, the maximal fluorescence change, corresponds to complete saturation by the ligands, and ΔF is the actual fluorescence change, obtained at a given concentration of ligand. A crucial assumption in our model is that the "free" ligand molecules that are in equilibrium with the binding site are, in fact, adsorbed somewhere on the protein surface, which would then mean that *one is, in fact, observing a kind of surface reaction*. To give this picture further support, we note that the association rates of, for example, AChE inhibitors can be extremely large ($\sim 10^9\ M^{-1}\ sec^{-1}$) and, therefore, are dominated by the initial encounter [9, 25]. Binding at a site inside a deep narrow channel cannot be expected to occur at such a high rate. It follows, therefore, that ligands must reach the binding site by means of diffusion on the macromolecular surface. In the binding of dopaminergic and β-adrenergic ligands, the equilibrium is reached in minutes [23, 26]. If association rates similar to those quoted above are assumed, corresponding to the association time scale of fractions of a second, we again have to assume that the binding site inside the transmembrane channel must be reached by a diffusion process through the membrane–receptor system. On the time scale of the binding constant measurements in these systems, the association (adsorption) takes place almost instantaneously, which means that we are, in fact, observing the binding process of ligands after they are "incorporated" into the membrane–receptor system.

CONCLUSIONS

Our analysis of the experimental facts about the four cases of the temperature-nearly-independent binding leads to the following binding model: After an almost instantaneous (on the time scale of the experiments) association, i.e. adsorption of the ligands to the macromolecular or membrane surface, the ligands reach, by surface diffusion, the binding site in the binding pocket located in a sterically constrained hydrophobic environment. The binding experiments in this case essentially reflect the equilibration between the ligands at the binding site and those in its hydrophobic surroundings. K_D, therefore, does not show the strong temperature dependence of ΔS^0 and ΔH^0 characteristic of hydrophobic interactions. The entropy increase that drives the temperature-nearly-independent binding ($\Delta H^0 \sim 0$) should arise from an increase of rotational phase space in the pocket (loose bond). If the ligand forms a tight bond, then $\Delta H^0 < 0$, but ΔH^0 and ΔS^0 depend only weakly on the temperature, and the van't Hoff plots are essentially straight. However, if the binding site is directly accessible to the bulk water, we can expect rather strong temperature dependence of ΔH^0 and ΔS^0, reflecting various degrees of participation of hydrophobic interactions. The van't Hoff plots are then more or less curved. This seems to be the case with the dopaminergic ligands.

The entropy-driven binding mechanism presently discussed involves interactions that are highly non-specific. It may therefore be considered in dealing with rather special practical problems, e.g. when we seek ligands for a binding site whose structure often mutates.

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