Thermodynamics of Ras/Effector and Cdc42/Effector Interactions Probed by Isothermal Titration Calorimetry*

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Proliferation, differentiation, and morphology of eucaryotic cells is regulated by a large network of signaling molecules. Among the major players are members of the Ras and Rho/Rac subfamilies of small GTPases that bind to different sets of effector proteins. Recognition of multiple effectors is important for communicating signals into different pathways, leading to the question of how an individual GTPase achieves tight binding to diverse targets. To understand the observed specificity, detailed information about binding energetics is expected to complement the information gained from the three-dimensional structures of GTPase/effector protein complexes. Here, the thermodynamics of the interaction of four closely related members of the Ras subfamily with four different effectors and, additionally, the more distantly related Cdc42/WASP couple were quantified by means of isothermal titration calorimetry. The heat capacity changes upon complex formation were rationalized in light of the GTPase/effector complex structures. Changes in enthalpy, entropy, and heat capacity of association with various Ras proteins are similar for the same effector. In contrast, although the structures of the Ras-binding domains are similar, the thermodynamics of the Ras/Raf and Ras/Ral guanine nucleotide dissociation stimulator interactions are quite different. The energy profile of the Cdc42/WASP interaction is similar to Ras/Ral guanine nucleotide dissociation stimulator interaction, despite largely different structures and interface areas of the complexes. Water molecules in the interface cannot fully account for the observed discrepancy but may explain the large range of Ras/effector binding specificity. The differences in the thermodynamic parameters, particularly the entropy changes, could help in the design of effector-specific inhibitors that selectively block a single pathway.

Ras plays a central role in cellular signal transduction by distributing many different extracellular signals into distinct cellular responses through activation of various downstream pathways (1). In its resting state, Ras is in a GDP-bound form and becomes activated by stimulated exchange of GDP for GTP (2). The GTP form of Ras binds more tightly to the so-called effector molecules than the GDP form and as a result of this interaction initiates, in the case of Raf, a protein phosphorylation cascade (3) that eventually leads to cell proliferation. Alternatively, other activities may be induced, such as RalGDS (4) or phosphatidylinositol 3-kinase (5). Using pull-down or two-hybrid assays, many other proteins were identified as Ras targets by their GTP-dependent association, all of which are sensitive to mutations in the effector-binding region of Ras (6). However, for many of these Ras targets an assigned biological function is yet to be determined. For instance, no sequence homology and no functional relationship has been ascribed to the putative Raf effectors AF6 (7) and Byr2 (8), the Schizosaccharomyces pombe homologue of the mammalian MEKK1 (9). Another avenue of the signals distributed by Ras leads to the Rho family of GTPases that consists of the members Rac, Rho, and Cdc42. These proteins also control cell proliferation but additionally are involved in the regulation of the NADPH oxidase complex (10) and the reorganization of the actin cytoskeleton (11–13). A key step in the latter process is the interaction of Cdc42 with the Wiskott-Aldrich syndrome protein WASP (14–16).

The three-dimensional structures of the RBDs of the effectors Raf and RalGDS were first determined by NMR spectroscopy and x-ray crystallography, respectively, and revealed the same ubiquitin-like fold for both domains (17–19). The crystal structures of the Ras/RalGDS-RBD and the Rap/Raf-RBD complexes have been solved (20–23). Besides a 30° rotation of the RBD relative to Ras or Rap, the mode of binding to a Ras protein is similar for Raf- and RalGDS-RBD in both complexes. The contacting areas of the molecules are anti-parallel β-strands that are held together by interactions of predominantly hydrophilic amino acid side chains (Fig. 1). Whether this represents a paradigm for Ras/effector interactions is currently investigated by the structural determination of the RBDs of Byr2 and AF6 (24, 25). In contrast, the contact area of the Cdc42/WASP complex contains predominantly hydrophobic side chains, and it is much larger than in the Ras/effector complexes (Fig. 1) (26).

Rap, TC21, and R-Ras are closely related to Ras and similarly interact with the Ras effectors in a GTP-dependent fashion. However, activation of enzymatic activity either could not be demonstrated or is much smaller than that induced by Ras. The interactions of Ras proteins with some of the effectors show large differences in individual affinities (27, 28). Intriguingly, Rap binds to some of the effectors more tightly than Ras, but

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1 The abbreviations used are: RalGDS, Ral guanine nucleotide dissociation stimulator; GDI, guanine nucleotide dissociation inhibition; GppNHp, guanosine 5′-β,γ-imido-triphosphate; mant, N-methylanthraniloyl, a fluorophor attached to the 2′ or 3′ position of the nucleotide; ITC, isothermal titration calorimetry; MEKK, mitogen-activated protein kinase kinase kinase; GppNHp, guanosine 5′-β,γ-imido-triphosphate; mant, N-methylanthraniloyl, a fluorophor attached to the 2′ or 3′ position of the nucleotide; ITC, isothermal titration calorimetry; MEKK, mitogen-activated protein kinase kinase kinase; RBD, Ras-binding domain; WASP, Wiskott-Aldrich syndrome protein.
apparently this does not result in correspondingly similar biological activities (29). Details about the effector binding of Ras proteins are known from biochemical and structural studies, but the differences in their biological functions could not be rationalized at the molecular level. To learn more about the origin of effector specificity for small GTP-binding proteins of the Ras subfamily, we investigated the thermodynamic properties of these interactions by means of isothermal titration calorimetry (ITC) and compared them to the thermodynamic characteristics of the Cdc42/WASP system. ITC is uniquely suited for this study because it directly measures the enthalpy of association, \( \Delta H \), and the affinity constant, \( K_a \) (30). Striking differences in the thermodynamic parameters are observed even with the closely homologous complexes of Ras-related proteins and effector RBDs that are difficult to reconcile with their structural similarities. The role of water in the protein/protein interface that provides Ras proteins with a wide range of binding specificity and possible implications for its biological functions are discussed.

**EXPERIMENTAL PROCEDURES**

Protein Purification and Nucleotide Exchange—Ha-Ras and Rap1A used in this work are termed Ras and Rap, respectively. Ras and Rap were produced in *Escherichia coli* CK5000K, whereas for all other proteins *E. coli* BL-21 was used. Ras and Rap were cloned in ptac, and in the case of Rap, pGRO/EL, encoding the *E. coli* Hsp60 chaperonin, was co-transformed into the cells. TC21 was cloned in pGEX 2T and R-Ras in the pET 11a plasmid. Only the RBDs of the effectors were used and purified as described: Rap-RBD corresponding to residues 790–886 cloned in pGEX 4T3 (27), AF6-RBD corresponding to residues 222–257 and 222–310, respectively. Cdc42 and W4 were purified, and bound nucleotide was exchanged as described (27). It is predominantly hydrophobic in the Cdc42/WASP complex, whereas the Rap/Raf-RBD complex has a strongly charged interface. The figure was prepared with GRASP (53).

**FIG. 1. Interface areas of effector complexes.** The figure illustrates the different modes of binding of the Rap/Raf-RBD complex (upper panel) and the Cdc42-WASP complex (lower panel). Raf-RBD and WASP are shown in green as backbone worms, and the GTP-binding proteins as molecular surfaces are colored according to their electrostatic potential. The boundaries of the effector contact area are marked by light green lines. In contrast to Raf-RBD, which displays a rather flat interface, WASP wraps around the GTP-binding protein in a largely extended conformation. The buried surface area is 1249 Å² in the Rap/Raf-RBD complex and 2939 Å² in the Cdc42/WASP complex. It is predominately hydrophobic in the Cdc42/WASP complex, whereas the Rap/Raf-RBD complex has a strongly charged interface. The figure was prepared with GRASP (53).

\[ K_a = \frac{[GTPase][effector]}{[GTPase \cdot effector]} \]

where the secondary ligand was injected into buffer alone. The back- change leveled off to a constant background value because of dilution processes were reflected by negative and positive power pulses, respectively. Saturation of the complex formation was reached when the heat change leveled off to a constant background value because of dilution effects. This background was also measured in control experiments where the secondary ligand was injected into buffer alone. The background was subtracted from the integrated pulses, and the data were analyzed using the manufacturer’s software yielding the stoichiometry (N), the binary equilibrium association constant (\( K_a \), which is equal to [GTPase-GppNHp effector]/[GTPase-GppNHp]/[effector] and equal to \( K_o^{-1} \)), and the enthalpy of binding (\( \Delta H \)). The experimental error on \( \Delta H \) is 5–10% and is predominantly governed by the error on the protein concentration in the syringe, whereas the experimental error on \( \Delta G^\circ \) is 0.2 kcal/mol, corresponding to an error of 40% on \( K_a \). The upper limit of reliable determination of \( K_o \) is \( 10^8 \) M⁻¹. The data were averaged over two or three ITC experiments. The result for \( N \) was 1.0 ± 0.2 for all measurements.
ments were done in 50 mM Tris/HCl, pH 7.4, 5 mM MgCl₂ (a), observed rate constants for the dissociation of Ras-mantGppNHp at 37 °C as a function of the RalGDS-RBD concentration. The fitted curves yield $K_d = 2.4 \mu M$ (○) and $K_d = 1.2 \mu M$ (□), and only relative, not absolute comparison is made. Additionally, no chemical modification of the system under study is necessary. To compare the results from ITC experiments with those obtained by the GDI assay, we derived binding constants using the enthalpy change $\Delta H^0$ for ITC, thereby possibly altering the thermodynamics of their interaction. This was tested for the Ras/RalGDS interaction in which both fluorescence and calorimetric titrations reveal a 7-fold lower affinity of the WASP fragments for Cdc42-mantGppNHp than for Cdc42-GppNHp (see Table II). WASP exhibits no significant GDI effect on Cdc42 but upon binding decreases the Cdc42/WASP interaction (33), which contrasts the Ras-effector interactions. As a result, the mant group does not seem to interfere with Ras-effector binding but does interfere in the Cdc42/WASP system. Proteins may form oligomers at the concentrations needed for ITC, thereby possibly altering the thermodynamics of their interaction. This was tested for the Ras/RalGDS-RBD interaction at two different effector concentrations (6 and 50 μM; Fig. 3). As anticipated for the absence of oligomerization, the results for $\Delta H^0$ and $K_d$ are almost identical, albeit less accurate in the case of the lower effector concentration. Thus, within experimental error, the results do not depend on the concentrations used, which excludes the possibility of protein oligomerization. This finding is corroborated by the fact that all proteins used here eluted as monomers in gel filtration studies. Also, swapping of the primary and secondary ligand yielded the same results for $\Delta H^0$ and $K_d$ (data not shown).

Proton release or uptake by amino acid side chains or the protein termini upon association leads to additional enthalpy effects and can be evaluated by performing ITC in buffers with different protonation enthalpies (36–39). The protonation enthalpies for Tris and phosphate are $-11.4$ and $-0.8$ kcal/mol (40), respectively, and this large difference should reliably allow the detection of a proton transfer process. To determine whether protons are exchanged during Ras-effector interactions and thereby influence the effector specificity of Ras, the
Binding of Ras to all four effector RBDs was measured in both 50 mM sodium phosphate, pH 7.4, 5 mM MgCl₂, 100 mM NaCl, and 50 mM Tris/HCl, pH 7.4, 5 mM MgCl₂, 100 mM NaCl (Table I). Within experimental error, ∆H° was buffer-independent for Raf/RBD, Byr2/RBD, and AF6/RBD (Table I). The slopes of the linear regressions yield the ∆C_p° values listed in Table I.

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Enthalpy and Entropy Contributions of GTPase/Effector Binding

To quantify the enthalpic and entropic contributions to the association reaction of small GTPases with their effectors, the interactions of Ras, Rap, TC21, and R-Ras with the RBD of Raf, RafGDS, Byr2, and AF6 were chosen. Additionally, the complex formation of Cdc42, a small GTPase of the Rho subfamily, with its effector WASP was investigated.

The Ras Subfamily and Its Effectors—Table I shows the primary results of the ITC experiments, K_a and ∆H°, together with the calculated ∆S° values. The binding constants observed and the resulting specificities for the different Ras proteins are similar to data obtained earlier by means of the GDI method (27, 28). Raf/RBD binds most strongly to Ras, whereas AF6- and RafGDS-RBD bind more strongly to Rap. The binding of Byr2 to the Ras proteins is less differentiated, whereas TC21 and R-Ras have similar and moderate effector affinities.

The comparison of the Ras proteins shows moderate differences in the thermodynamic constants. The weaker binding of Raf/RBD to Rap compared with Ras is due to a smaller enthalpy change, whereas the entropy changes are similar. The lower affinities of TC21 and R-Ras for Raf-RBD are due to less positive entropy changes with the enthalpy changes being the same. The affinities of Rap for Raf-RBD and RafGDS-RBD are in inverse to those of Ras, and the stronger binding of RafGDS-RBD to Rap compared with Ras is the result of a less unfavorable entropy change, whereas the enthalpy change stays the same. TC21 and R-Ras show large differences of ∆S° and ∆H° as compared with Ras that compensate each other almost entirely and lead to very similar affinities for RafGDS-RBD as Ras. The binding affinities to Byr2 are not significantly different for Ras and Rap, and the enthalpy and entropy changes are the same and close to zero. By contrast, significant differences in Ras and Rap binding to AF6 are observed. ∆H° for Rap is less favorable by 6.1 kcal/mol, but ∆S° is increased by 7.7 kcal/mol, which increases K_a 15-fold.

The binding of Raf/RBD to the Ras proteins is achieved to a similar extent by both favorable enthalpy and entropy changes. In contrast, the interaction of RafGDS-RBD with the Ras proteins results from an unfavorable entropy change that is counteracted by a large negative change in enthalpy. Byr2 and AF6 exhibit similar enthalpy and entropy changes upon GTPase binding and lie in between the two extremes Raf and RafGDS; their binding to Ras is mediated by a medium-sized decrease in enthalpy, whereas the entropy change is close to zero.

One of the most meaningful thermodynamic parameters in terms of characterizing the nature of protein/protein interactions is the change in heat capacity, ∆C_p° (41–43). Provided the temperature dependence of ∆H° is linear over the temperature range studied, ∆C_p° is obtained as the slope of a plot of ∆H° versus the temperature. Fig. 4a shows calorimetric titrations...
for the Ras/Byr2 system at four different temperatures, and from Fig. 4b the linear dependence of $\Delta H^o$ on temperature for the interaction of all four effector RBDs with Ras is evident. All protein complexes studied exhibit a decrease in heat capacity upon association (Tables I and II), indicating that the complex buries hydrophobic areas. As with the observations on $\Delta H^o$ and $\Delta S^o$, the values of $\Delta C_p^0$ are largely different between the effectors, whereas similar results are obtained for both Ras and Rap. No apparent correlation exists between the $\Delta C_p^0$ and $\Delta H^o$ or $\Delta S^o$ values.

The Rho Subfamily: Cdc42 and WASP—To compare the thermodynamic data of Ras and effector proteins to a more distantly related GTPase/effector couple, the interaction of Cdc42, a member of the Rho/Rac family of small GTPases, with two different fragments of its effector WASP was investigated. Although the RBD of Rap effectors is compactly folded, a short, unstructured 4-kDa sequence (W4) in WASP containing the Cdc42/Rac interactive binding motif is sufficient for Cdc42 binding (33). Yet a 10-kDa fragment (W10) binds even more tightly (Table II). Effector-type binding of W10 is evidenced by a 470-fold difference in the affinities for the GDP- and GppNHp-bound form of Cdc42. The affinity of Cdc42 and WASP is at the high end compared with the Ras and effector proteins. Similar to the Ras/RalGDS complex, Cdc42 binding to WASP is strongly favored by a large negative enthalpy change that overcompensates for a negative entropy change. Intriguingly, there is a large difference in the enthalpy and entropy contributions for the W4 and W10 fragments. Whereas the enthalpy change becomes less favorable by 5.1 kcal/mol upon extension of W4 to W10, the entropy change becomes less unfavorable by 6.2 kcal/mol, which leads to the 7-fold stronger binding of W10. This large difference in entropy may reflect the ordering of the Cdc42/Rac interactive binding motif in W10 in a binding-competent conformation that is not present in W4. Indeed, the circular dichroism spectra of W4 and W10 reveal a significant amount of secondary structure in W10 but not in W4 (data not shown).

Comparing the GTP and GDP complex of Cdc42 binding to W10, the 470-fold drop in affinity is almost exclusively due to a less favorable enthalpy change of 3.8 kcal/mol for the GDP form, whereas the entropic contribution to binding is virtually unaltered. This could be interpreted as a structural difference between the GTP and GDP states of Cdc42 that does not affect the size of the interface area but only the intermolecular distance or particular conformations of the side chains involved. This would lead to impaired hydrogen bond or salt bridge formation between residues in the Cdc42-GDP/W10 complex. Alternatively, Cdc42-GDP may bind WASP in a manner similar to Cdc42-GTP, but extra energy is required to convert it to the GTP-like conformation.

In contrast to the interaction of Ras and Rap with their respective effectors, where the presence of 100 mM NaCl increases $K_b$ by a factor of 2 or more, the Cdc42/WASP interaction is not influenced by even the presence of 2 mM NaCl (data not shown). This indicates that the interaction between Cdc42 and its effector WASP is of a strongly hydrophobic nature, whereas the Ras and Rap interaction with their effectors has strong electrostatic components.

Mutants of Ras and Raf-RBD—From the comparison of the GTPase/effector systems, it is apparent that enthalpy and entropy changes are not directly evident from the high resolution crystal structures of the protein complexes. Even closely related proteins, as investigated here, show large variations in their thermodynamic parameters. To test whether single amino acid residues are responsible for this, we extended our studies using mutants of Ras and Raf-RBD. Residues to be mutated were selected among those that are involved in the interaction. Table III collects the $\Delta H^o$ and $\Delta S^o$ values for mutants that bind to wild type Ras and vice versa for Raf mutants that bind to Ras. Relatively small changes in comparison with the wild type proteins are observed. No mutation causes a change of more than 2 kcal/mol in $\Delta H^o$ or $\Delta S^o$. A clearly consistent effect of the elimination of a distinct residue on $\Delta H^o$ or $\Delta S^o$ cannot be recognized among this limited number of mutants. In conclusion, the differences in binding energetics observed for the different effectors are much larger than effects that can be evoked by single residues.

Comparison of Measured and Calculated Heat Capacity of Binding

From structural and protein folding studies, an empirical relationship between the heat capacity of folding $\Delta C_p^0$ and the change in the accessible surface area ($\Delta A_{ASA}$) was derived (44) as follows.

$$\Delta C_p^0 = 0.45 \text{cal/(mol KÅ)}^2 \triangle A_{ASA \text{polar}}$$

$$- 0.26 \text{cal/(mol KÅ)}^2 \triangle A_{ASA \text{polar}}$$ (Eq. 1)

The apolar surface area that becomes buried in the interior of the protein on folding results in a negative change of the heat capacity, whereas polar amino acid residues have a positive contribution that is smaller in amount. Protein folding and protein/protein association may be regarded as related because in both processes surface area is buried in the interior of a protein or a protein complex, respectively. For some of the Rasseffectors complexes, the three-dimensional structures are known, namely for Rap/Raf-RBD (20), for Rap(E30D/K31E)/Raf-RBD (21), for Ras(E31K)/RalGDS-RBD (22), and for Ras/ RalGDS-RBD (23).
The ΔASA values for the three complexes were calculated on the assumption that complex formation does not induce large conformational changes that may lead to additional changes of the accessible surface area outside the interface (Table IV). The interface areas are similar in size, and the polar areas are about twice as large as the apolar areas (Fig. 1 and Table IV). A similar number of polar and apolar amino acid side chain interactions constitutes the complex interface. This lead to calculated ΔC_p^0 values for all complexes close to 0 that do not agree with the experimental results (Table IV). Whereas in protein folding essentially all solvent molecules are stripped off the protein surface to become part of the protein core, many examples are known where water molecules are bound at complex interfaces. Consequently, the interface area of the Ras/effector complexes may not be completely desolvated, and the number of water molecules needs to be taken into account for the calculation of ΔC_p^0. The crystal structures of Rap/Raf-RBD and Ras/RalGDS-RBD show water molecules that bridge amino acid residues of the interacting proteins by simultaneously forming hydrogen bonds to both proteins. Although different numbers of such water molecules are observed in the different complexes (Table IV), they cannot by far account for the different experimental ΔC_p^0 values or for the discrepancy to the calculated values. In addition, the different temperatures of x-ray data collection (277K for the Raf-RBD complexes and 92K for the RalGDS-RBD complex) and the different resolution ranges of the structures (1.8–2.1 Å) have to be considered, which may lead to the different numbers of water molecules found in the structures. Interestingly, for the Cdc42/WASP complex the measured and calculated ΔC_p^0 values are very similar (Table IV). The hydrophobic nature of this interface may prevent water molecules from being trapped upon complex formation. Thus, the equation for the calculation of ΔC_p^0 from the interprotein surface areas may be useful only for complex interfaces that are highly hydrophobic in nature.

**DISCUSSION**

**Table IV**

*Structural observations of the available Ras/effector complex structures*

Calculations were done on the structures of Rap • Raf-RBD (20), Rap(E30D,K31E) • Raf-RBD (termed Raps • Raf) (21), Ras(E31K) • RalGDS (22), and Cdc42 • WASP (26).

| Calculated ΔC_p^0 (cal/mol/K) | Measured ΔC_p^0 (cal/mol/K) |
|-------------------------------|-----------------------------|
| Rap • Raf                     | 37                          | -300                        |
| Raps • Raf                    | 79                          | -270                        |
| Ras • RalGDS                  | -5                          | -630                        |
| Cdc42 • WASP                  | -594                        | -530                        |

* According to Equation 1.

The interaction of Ras with various effectors is essential for signal transduction into different pathways and is the basis for its high functional diversity. However, the biological importance of the closely related Ras proteins that bind to the same effectors still remains elusive. The effectors differ both in their functions and in their primary sequences, but it appears that a small domain in each of them, 80–140 amino acid residues in size and located in different parts of the effector, is responsible for Ras binding. Similarly, a sequence in WASP is responsible for its interaction with Cdc42 (33, 45), and in this study a 4- and a 10-kDa fragment were used. For the interactions of Ras proteins and effectors very different affinities were reported that span 3 orders of magnitude and thereby indicate a possible biological significance (27, 28). In addition, the three-dimensional structures of RalGDS- and Raf-RBD revealed an identical fold and a docking similar to that of the Ras protein (20–23).

Here, we wanted to characterize the underlying binding energetics by quantifying the enthalpic and entropic contributions to the GTPase/effector association reactions. This should provide information about the type of binding that can be grouped in a first approach into hydrophilic and hydrophobic interactions. The latter are believed to result predominantly in favorable entropy changes because strongly constrained water molecules from the surface of hydrophobic amino acid side chains are released into bulk water on complex formation (41). In contrast, the change in entropy caused by contact formation between hydrophilic residues should be small because the number of hydrogen bonds including inter-water binding does not change (46). However, the global conformational changes and the restriction of side chain mobility may substantially contribute to a decrease in entropy (see below). Although desolvation is entropically favored, a penalty in enthalpy must be paid that needs to be counterbalanced by formation of polar interactions such as hydrogen bonds and salt bridges. A priori it is difficult to predict the net energy outcome because many single molecular events with large individual changes in enthalpy and entropy contribute to the association process (46). These for the most part compensate each other and result in relatively small ΔH^0 and ΔS^0 values. We have tried to investigate to what extent the high homology among the Ras proteins and the same topology of Rap-RBD and RalGDS-RBD is reflected in the similarity of their binding energy profile and compared them to a different type of small GTPase/effector interaction, the Cdc42/WASP complex.

Considering the structural similarities of Rap- and RalGDS-RBD and their similar type of interaction with the Ras proteins (20–23) with respect to the number of hydrogen bonds and salt bridges and the size of the hydrophobic interface area, more similar thermodynamic parameters might have been expected. The opposite sign in the change of entropy for Raf and RalGDS binding, which leads to a difference of more than 10 kcal/mol in ΔS^0, is an especially intriguing finding and difficult to explain from the structures. We can exclude proton transfer reactions from being responsible for the difference in the thermodynamic constants, because only a small buffer-dependent effect on ΔH^0 was visible for Rap-RBD. No single amino acid residue causes the large differences either because mutants resulted only in moderate changes of ΔH^0 and ΔS^0. Rather, the large differences in ΔH^0 and ΔS^0 for the interaction of Ras proteins with Raf and RalGDS might indicate a different mode of binding that is not obvious from the three-dimensional structure.

The hydrophobic effect, commonly believed to increase the entropy, cannot account for this difference because the apolar interface area in the Rap/Raf complex is even slightly smaller than in the Ras/RalGDS complex. The maximum gain in entropy by the transfer of protein-bound water to bulk solution is 7 cal/(mol K) corresponding to TΔS^0 = 2 kcal/mol at 25 °C (47).
If the strongly bound water molecules were responsible for the observed entropy effects, Raf binding should release at least six water molecules more than RaIGDS binding. This seems unlikely, because there are no clefs or other sites on the Raf-RBD surface that could fix water. In fact, the interface volumes of the x-ray structures suggest the same number of water molecules in the Rap/Raf and Ras/RaIGDS complexes (21, 22).

Another source of entropy change is the loss of side chain flexibility or conformational freedom of the protein backbone. Comparison of the RBD structures in the free state (17–19) and in the complexes reveals no major structural rearrangements. Huang et al. (22) suggest that a conformational difference between free and complexed RaIGDS-RBD, localized in loops β1/β2 and α1/β3, might play a role in the activation of the catalytic domain. However, the Ca root mean square deviation of 1.3 Å is not exceedingly large. A decrease in entropy as observed here for Ras/RaIGDS binding would support this notion if this structural rearrangement resulted in decreased conformational freedom of the complex. However, this evidence is weak, and the structures of the free and complexed proteins do not have significantly different temperature factors indicating similar main chain mobility. It is difficult to quantitatively relate structural to entropy changes, and here not even the large differences in $\Delta S^0$ between Raf- and RaIGDS-RBD can be attributed to obvious structural differences. In conclusion, subtle conformational changes of amino acid side chains or loop regions or incorporation of solvent molecules into the binding site may lead to the large observed differences in entropy. Additionally, a change in hydration of bound metal ions can also contribute to the $\Delta S^0$ value, but a different influence of the effectors on the environment of the nucleotide-bound Mg$^{2+}$ ion seems to be unlikely.

The heat capacity changes are also different for all effectors and are not reconcilable with the empirical relationship between $\Delta C_p^0$ and the burial of surface area in protein folding. A more refined set of parameters taking a different contribution of Ser and Thr into account (43, 48) or using a set of parameters more refined set of parameters taking a different contribution of Ser and Thr into account (43, 48) or using a set of parameters strongly resemble the values obtained for the interaction of effectors on the environment of the nucleotide-bound Mg$^{2+}$ ion. Additional evidence, D. Klostermeier for helpful comments on the manuscript, K. P. Hofmann and G. Weiss for initial help with calorimetric measurements, and H. Tu and M. Wigler for providing the Byr2 DNA construct.

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