Substrate and Product Structural Requirements for Binding of Nucleotides to H-ras p21: The Mechanism of Discrimination between Guanosine and Adenosine Nucleotides†

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Received August 31, 1994; Revised Manuscript Received October 14, 1994®

ABSTRACT: The interaction of the protein product of the H-ras oncogene with a series of nucleoside di- and triphosphates has been examined to investigate the tolerance of the active site to departures from the GTP or GDP structures. Nucleotides which bind relatively strongly could be used as competitors of GDP in a simple filter binding assay to give semiquantitave estimates of their affinities. For more weakly binding nucleotides or to obtain quantitative data, a transient kinetic method was used which was based on determination of the association and dissociation rate constants. The results obtained indicate that substantial modification of the sugar or phosphate structure is tolerated with little or moderate loss of affinity, but that large losses in affinity occur on modification of the base structure. In particular, replacing the guanine by an adenine residue leads to a dramatic loss of affinity. Thus, discrimination against ATP and ADP is very high (relative affinities of ATP and GTP $1:10^7$). This is due not only to loss of positive (stabilizing) interactions, but especially to the introduction of negative ones.

GTP-binding proteins, such as the heterotrimeric G-proteins, ribosomal initiation and elongation factors, and the small GTP-binding proteins related to the ras oncogene product, all bind GTP and GDP with high affinity and high specificity with respect to the base. While quantitative data are difficult to find in the literature, there appears to be dramatic discrimination against adenine, but less against inosine (Sigal et al., 1986a,b; Noonan et al., 1991). While the significance of this differentiation is apparent, it is of interest to investigate the mechanism of the effect. H-ras p21[1] provides an opportunity to do this, since it is available in large amounts from expression in bacteria, and the structure is known in great detail (de Vos et al., 1988; Pai et al., 1989, 1990; Schlichting et al., 1990; Milburn et al., 1990). We have addressed this question by comparing the interaction of GTP (or GDP) and other nucleotides with p21 in the present paper. To do this accurately, especially in the case of weakly bound nucleotides, it was neccessary to use transient kinetic methods in conjunction with fluorescent derivatives of guanosine nucleotides or a fluorescent mutant of p21. The data obtained are significant for understanding the mechanism of the p21 GTPase reaction, in particular the mechanism of discrimination between GTP and other naturally occurring nucleotides, and provide information on the design of modified nucleotides as tools for probing reactions involving GTP-binding proteins as well as for considerations of the potential of nucleoside (or nucleotide) analogs in drug development studies.

MATERIALS AND METHODS

p21 was prepared as previously described using an *Escherichia coli* expression system (Tucker at al., 1986). The preparation and characterization of the Y32W mutant will be described elsewhere (Lautwein et al., in preparation). Nucleotide-free p21 was prepared as described (John et al., 1990). *N*-Methylanthraniloyl derivatives of GDP, GTP, dGDP, dGTP, GPPNP, and dGPPNP were prepared essentially as described (John et al., 1990). Other nucleoside triphosphates were prepared from the parent nucleoside essentially as described for the one-vessel synthesis of nucleoside α-thiotriphosphates (Goody & Isakov, 1984), except that phosphoryl chloride was used instead of thiophosphoryl chloride in the first step. Purification was by standard ion-exchange and reverse-phase methods as described in Goody and Isakov (1984).

Filter-binding assays to monitor the interaction of [^{3}H]GDP with p21 were performed as previously described (Tucker et al., 1986). Kinetic data were analyzed by nonlinear regression, normally using a single exponential function, with the program Enzfitter (Elsevier Biosoft) or Grafit (Erithacus Software).

Static and slow time scale dynamic fluorescent measurements were done using an SLM 8000 spectrophotometer. Excitation and emission wavelengths were normally 360 and 440 nm for methylanthraniloyl derivatives or 280 and 340 nm for the Y32W mutant, respectively. Nucleotides were separated analytically by reversed-phase HPLC as described

† The financial support of the Deutsche Forschungsgemeinschaft is greatfully acknowledged.

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® Abstract published in *Advance ACS Abstracts,* December 1, 1994.

[1] Abbreviations: p21, product of the H-ras protooncogene; mant, methylanthraniloyl group; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); GppNHp, guanosine 5′-*O*-(β,γ-imidotriphosphate); $GppCH_2p$, guanosine 5′-*O*-(β,γ-methylenetriphosphate).

0006-2960/95/0434-593$09.00/0

Table 1: Effect of Phosphate Structure or Presence on the Affinity of Nucleoside Triphosphates to p21[a]

nucleot(s)ide	affinity relative to GDP
GTP	1.9
GppNHp	9×10^{-2}
GppCH$_2$p	1×10^{-2}
GMP	7×10^{-7} [b]
guanosine	1.6×10^{-7} [b]

[a] The relative affinity is expressed as the ratio of the association constant of the nucleotide to that of GDP. [b] Determined by the stopped-flow method; all others by filter binding.

previously (Rensland et al., 1991) using a UV absorption detector at 252 nm.

Stopped-flow experiments using methylanthraniloyl nucleotides were performed as described (John et al., 1990). Similar experiments with the Y32W mutant of p21 made use of the fact that there is an increase in tryptophan fluorescence intensity (ca. 60%) on binding of GDP, IDP, or ADP to the protein.

RESULTS AND DISCUSSION

As pointed out recently, the affinity of guanosine nucleotides for p21 and many other GTP/GDP-binding proteins cannot be determined conveniently by classical titration procedures because of the combined and related effects of very high affinity and the presence of a stoichiometric amount of GDP on the protein when isolated in the normal manner (Goody et al., 1991). Affinities of nucleotides relative to that of GDP can be obtained, in principle, from competitive titrations of the nucleotides to the complex between p21 and GDP as it is normally isolated. This method fails for very weakly bound nucleotides, and has another major drawback, which arises from the extremely slow rate of dissociation of the GDP. The rate of dissociation can be accelerated significantly by removal of magnesium ions, and experiments reported here and previously on relative affinities of nucleotides to p21 have normally involved incubation of the 1:1 complex of p21 and GDP with the competing nucleotide in the presence of EDTA, followed by addition of excess Mg^{2+} before quantitating the amount of GDP displaced by the competitor. It should be realized, however, that the relative affinity obtained by this method is actually that which applies in the absence of metal ions, since addition of Mg^{2+} merely serves to "freeze" the nucleotide at the active site for the period of the measurement procedure. Thus, accurate statements on the relative affinities are not to be expected from this method, but it is useful as a convenient way of examining the effect of structural modifications of guanosine nucleotides on their affinity in a semiquantitative manner. As explained below, a number of the more interesting nucleotides were also examined by a transient kinetic method, which leads to a reliable estimate of the affinities as well as of kinetic parameters of the nucleotides used (John et al., 1990).

Phosphate-Modified Analogs. The influence of modification of the phosphate groups is seen in the results given in Table 1. GTP binds somewhat more tightly than GDP, as reported earlier. Replacement of the β,γ-bridging oxygen by an NH group, leading to the nonhydrolyzed GTP analog GppNHp, leads to a drop in affinity of ca. 10 compared with GDP, or 20 compared with GTP. This reduction in affinity appears to be due to a weakening (for GppNHp) or loss (for GppCH2p) of a hydrogen bond between the β,γ-bridging group and the backbone NH of Gly13, an interaction seen in the 1.35 Å structure of p21·GppNHp (Pai et al., 1990). Replacement of the same group by the CH_2 group leads to a further drop in affinity by a factor of ca. 10, so that this modification reduces the affinity by a factor of 200 in comparison with GTP·caged GTP, which has a bulky group on the γ-phosphate that binds with relatively high affinity (Schlichting et al., 1990). The presence of the ester group on the γ-phosphate is probably tolerated because one of the oxygens of the γ-phosphate group is not involved in an interaction with the protein or Mg^{2+}·GTPγS is the best (in terms of affinity) phosphate-modified analog of GTP, which corresponds to general experience with GTP- and ATP-utilizing enzymes.

Table 2: Effect of Sugar Structure on the Affinity of Nucleoside Triphosphates to p21[a]

nucleotide	affinity relative to GDP	nucleotide	affinity relative to GDP
2′-dGDP	1.2	3′-NH$_2$-GTP	1.1
2′-dGTP	1.7	2′(3′)-mant-GDP	0.69
2′,3′-ddGTP	0.98	2′(3′)-mant-GTP	1.28
2′-OCH$_3$-GDP	0.71	3′-mant-2′-dGTP	0.92
3′-OCH$_3$-GDP	0.61	3′-Ac-2′-dGTP	0.14
3-OCH$_3$-GTP	0.79	ppGpp	0.56
3′-NH$_2$-GDP	1.7	pppGpp	0.25

[a] The relative affinity is expressed as the ratio of the association constant of the nucleotide to that of GDP.

Removal of both the β- and γ-phosphate groups from GTP to give GMP leads to a dramatic loss of affinity, which is probably at least partly associated with the loss of Mg^{2+}-binding properties (although GTP and GDP can still bind, with reduced affinity, in the absence of Mg^{2+}). The interaction of GMP and guanosine with p21 will be considered again below in connection with the weak binding of ATP.

Sugar-Modified Analogs. As shown in Table 2, extensive modifications at the 2′- and 3′-positions of the sugar residue of guanosine are tolerated with little loss in binding affinity of the nucleotides to p21. From the high-resolution structure of p21, it can be seen that there are weak interactions of the side chain of Asp-30 with the 2′- and 3′-hydroxyl groups, as well as an interaction with the carbonyl group of Val-29. These interactions appear to be energetically of little significance, since even removal of both hydroxyl groups only reduces the affinity by a factor of 3. Bulky groups are also tolerated, as seen in the case of the methylanthraniloyl esters. The structure of p21 with a 3′-methylanthraniloyl ester of 2′-deoxyGppNHp has recently been determined (Goody et al., 1992), and this shows that the mode of binding of the nucleotide part of the structure is almost identical with that of GppNHp. The methylanthraniloyl group has enough room to extend away from the sugar residue without interacting with or disturbing the protein structure significantly.

Base-Modified Analogs. The influence of chemical modification of the guanine residue of GDP or GTP on their interaction with p21 is of special interest because of the known high base specificity for this interaction. The results of the effects of 11 modifications are shown in Table 3, where the relative affinities with respect to GDP are collated. It can be seen on first inspection that modification of the

Table 3: Effect of Base Structure on the Affinity of Nucleoside Triphosphates to p21[a]

nucleotide	affinity relative to GDP	
	filter binding	stopped-flow
GTP	1.9	
ITP	10^{-3}	3.6×10^{-2}
XTP	6.9×10^{-3}	1.5×10^{-2}
ATP		4.2×10^{-7}
6-SH-GTP	1.3×10^{-2}	
Br-GTP	1.2×10^{-3}	
2-NH_2-PurTP	2.3×10^{-2}	
7-deaza-2′-dGTP	7.1×10^{-2}	
7-CH_3-GTP	7.4×10^{-3}	
N2-isobut-dGTP	0.29	
6-Cl-ITP	3.7×10^{-3}	
Purrib-TP	2.9×10^{-3}	

[a] The relative affinity is expressed as the ratio of the association constant of the nucleotide to that of GDP.

base structure is generally not well tolerated, suggesting that there are a number of energetically important interactions between the protein and the base. Examining the effects at individual positions separately, it can be seen from the data that the amino group at position 2 must be involved in an important interaction, since removing it leads to a ca. 100-fold loss of affinity (ITP). Examination of the high-resolution structure of p21·GppNHp (Pai et al., 1990) reveals that this is the interaction with an oxygen of the side chain of Asp-119, which is part of the highly conserved (in guanine nucleotide-binding proteins) ^{116}NKXD sequence. The other oxygen of the Asp-119 side chain forms a hydrogen bond with the hydrogen on N1 of the guanine ring in the p21·GppNHp structure, and this can, presumably, still occur with inosine, since the presence of the keto group at position 6 leads to the same tautomeric structure in this region for guanosine and inosine. Another important interaction with the base, that between the backbone NH of Ala-146 and the 6-keto group of guanosine, can also occur with inosine.

There is evidence from other studies that only one of the hydrogens of the 2-amino group is involved in an interaction with the protein. Thus, one of the hydrogens can be replaced by bulky groups without loss of affinity to p21 (Noonan et al., 1991).

Replacement of the keto group at position 6 of the purine function by a proton also leads to a ca. 100-fold drop in affinity (2-aminopurine riboside triphosphate; Table 2). Replacement by a thiol group at the 6-position has a similar effect. These reductions in binding energy must be due to loss of, or weakening of, the hydrogen bond to the main chain NH of Ala-146, and loss of the hydrogen bond between Asp-119 and the proton on N1 of the guanine ring system in the case of 2-aminopurine.

Replacement of N7 of the guanine ring by a carbon, as in 7-deazaguanine, leads to a reduction of affinity of ca. 50. This is due to loss of the hydrogen bond between N7 of the guanine ring and the side chain of the conserved residue Asn-116. Methylation at N7 reduces the affinity by a factor of ca. 500, possibly due to steric hindrance and to the introduction of a positive charge.

Weakly Bound Nucleotides. The method used for obtaining the results described above is not applicable to nucleotides which bind many orders of magnitude less strongly than GDP, since unrealistically high concentrations of the weakly bound nucleotide would be needed to displace the tightly bound GDP. For this reason, we resorted to a kinetic method to examine the interaction of p21 with very weakly bound ligands such as GMP and guanosine (John et al., 1991). It was shown several years ago that GTP carrying a fluorescent label on the ribose moiety (2′- and 3′-methylanthraniloyl esters) had good substrate properties with p21 (John, 1990; Neal et al., 1990). They were used to characterize the kinetics of binding of GTP and GDP, and these experiments led to the conclusion that the binding reaction is at least a two-step process for both nucleotides, with a weak but rapid initial binding being followed by a quasi-irreversible isomerization step. This leads to Scheme 1 for GTP binding to p21. In the case of GTP, K_1 has a value of 1.25×10^5 M^{-1}, k_{+2} a value of 21.8 s^{-1}, and k^{-2} a value of 2.9×10^{-5} s^{-1}.

Scheme 1: General Scheme for the Binding of Nucleotides to p21[a]

$$\text{p21} + \text{N} \underset{}{\overset{K_1}{\rightleftharpoons}} \text{p21•N} \overset{K_2}{\rightleftharpoons} \text{p21}^*\text{•N}$$

[a] N is any nucleotide which has affinity for the p21 active site.

The experiments leading to the formulation of Scheme 1 involved mixing of nucleotide-free p21 with fluorescent GDP or GTP analogs in a stopped-flow apparatus using the fluorescence change which occurs in the second step of the mechanism as a signal of binding. If a competing nucleotide is included in the substrate syringe of the stopped-flow apparatus, there will be an effect on the kinetics of binding of the fluorescent nucleotide to p21. Depending on the details of the kinetics of the interaction of the competing nucleotide with p21, different effects are seen on the binding transient of the fluorescent nucleotide. As described by John et al. (1990), for a competing nucleotide which binds relatively tightly to p21 (i.e., one for which $1/k_{-2}$ is small compared with the time scale of the association transient), increasing the concentration of the competing nucleotide at constant fluorescent nucleotide concentration leads to pseudo-first-order kinetics (assuming nucleotide is in excess of p21) with an increase in the rate constant of the fluorescent transient, and the two-step mechanism leads to saturation behavior; i.e., there is a hyperbolic dependence of the rate constant on the concentration of competitor, from which K_1 and k_{+2} can be extracted. In parallel to this increase in rate constant, there is a decrease in amplitude of the fluorescence change. This type of behavior is seen when GDP or GTP competes with mant-GDP. On the other hand, if the competitor is in very rapid equilibrium with its bound form on p21 (i.e., if k_{-2} is large or if the second step does not occur), increasing its concentration leads to pseudo-first-order kinetics with a hyperbolic decrease of the rate constant toward zero. If, as in the case of guanosine and GMP, the overall affinity is many orders of magnitude lower than for the fluorescent nucleotide, there is no effect of the competitor on the amplitude of the fluorescent signal. The results obtained by John et al. led to values of 153 and 29 μM for the dissociation constants of guanosine and GMP, repectively (cf. 10^{-11} M for GTP).

The method outlined here and described in more detail by John et al. (1991) was used in the present work to investigate two of the nucleotides already examined using the equilibrium competition method, namely, ITP and XTP. In addition, since we could find no evidence of ATP binding to p21 using the equilibrium assay described above, we used the kinetic method to obtain evidence of the interaction in

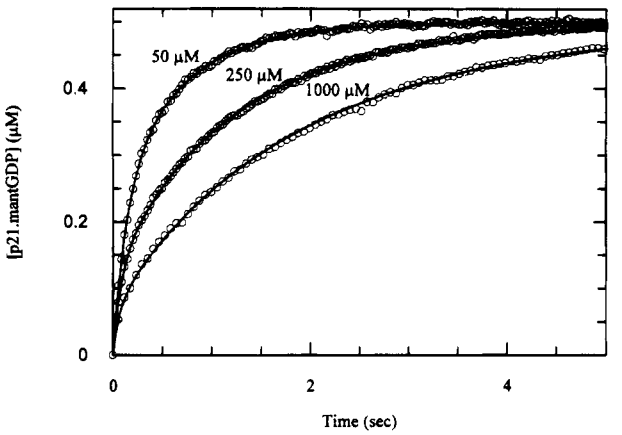

FIGURE 1: Association transients between mant-dGDP (1.5 μM) and p21 (0.5 μM) in the presence of 50, 250, or 1000 μM ATP. The fluorescence signal used in the stopped-flow apparatus to monitor the binding of mant-dGDP was converted into concentration units, and constants were fitted assuming a two-step binding mechanism for both nucleotides. The constants were fitted using a program which simulates the time course of the reaction using a numerical integration procedure and also provides a best fit (shown by the solid line) to the data. The fitted constants for ATP are shown in Table 4. The constants used for mant-dGDP (determined independently) were $K_1 = 2.2 \times 10^5$ M^{-1}, $k_{+2} = 22.8$ s^{-1}, and $k_{-2} = 7 \times 10^{-5}$ s^{-1}. The temperature was 25 °C, and the buffer conditions were 40 mM Hepes/NaOH (pH 7.5), 2 mM $MgCl_2$, and 1 mM DTE.

Table 4: Kinetics of Interaction of Nucleoside Triphosphates with p21[a]

nucleotide	K_1 (M^{-1})	k_{+2} (M^{-1} s^{-1})	k_{-2} (s^{-1})	K_{ass} (M^{-1})
GTP	1.25×10^5	21.8	1.7×10^{-5}	1.6×10^{11}
ATP	2.0×10^3	22.4	2.1	2.3×10^4
ITP	1.02×10^5	20.4	1.2×10^{-3}	1.7×10^9
XTP	6×10^4	18.4	1.5×10^{-3}	7.4×10^8

[a] Values obtained from stopped-flow experiments using mant-GDP and the respective nucleotide as described in the text. K_{ass} is the product of K_1 and $(1 + K_2)$, and is a measure of the overall affinity between the nucleotide and p21.

this case as well. Interestingly, ATP behaved quite differently to ITP and XTP. Whereas the latter two nucleotides showed behavior which was qualitatively and quantitatively similar to GTP or GDP in this kind of experiment, two differences were noted with ATP. First, at concentrations of ATP which were similar to those of the fluorescent nucleotide used (mant-dGDP), there was no noticeable effect on the fluorescence transient, suggesting that the affinity of p21 for ATP is indeed much lower than for guanosine nucleotides or even ITP or XTP. Second, on increasing the ATP concentration to 50 μM or above, there was a reduction in the rate of the transient, but in contrast to the situation with GMP or guanosine, there was marked deviation from an exponential time course, and at very high ATP concentrations (>1 mM), the rate did not tend toward zero. This behavior suggested that the kinetic characteristics of the p21−ATP interaction are intermediate between those for p21−GDP and p21−GMP. The data obtained were analyzed using a simulation/fit program based on numerical integration of the differential equations describing the time dependence of the interaction of p21 with mant-dGDP and ATP. As shown in Figure 1, good fits to the data could be obtained with the constants given in Table 4 and the legend to Figure 1. The value for k_{-2} for ATP from these fits was 3.1 s^{-1}, and this was checked in an experiment in which the p21 was first loaded with ATP, which was then displaced with mant-dGDP. This led to a value of 2.1 s^{-1}, which is reasonable agreement considering the fact that a large number of constants were fitted to the data in the simulation analysis. An additional check was provided by the following experiment. p21 was mixed with ATP at a concentration of 50 μM, after which it was displaced by mant-dGDP. The ensuing fluorescence transient showed two phases: a faster one corresponding to binding of the fluorescent nucleotide to free p21 and a slower one occurring at ca. 2 s^{-1} corresponding to the rate of ATP release. The relative amplitude of the two phases was ca. 1:1, meaning that at 50 μM, 50% production of the p21•ATP complex occurred, suggesting that the K_d value for ATP is ca. 50 μM. This is in reasonable agreement with the value of 44 μM for $1/[K_1(1 + K_2)]$ from Table 4.

Since these measurements were relatively indirect, we have extended them using a fluorescent mutant of p21 (Y32W). As will be described elsewhere, the tryptophan fluorescence yield of this mutant is increased significantly (by ca. 80%) on interaction with GDP, but not with GTP or its analogs. We have also determined the structure of this mutant, and shown that the substitution of tryptophan for tyrosine does not result in disturbance of the overall structure (Lautwein et al., in preparation). Using this mutant, it was possible to investigate the replacement of guanine by other bases in a direct manner using the nucleoside diphosphates (GDP, IDP, and ADP). As for GDP, binding of IDP or ADP to the mutant leads to a substantial increase in fluorescence yield. Figure 2 shows the dependence of the pseudo-first-order rate constant for binding of the three nucleotides to p21. The binding kinetics of IDP and GDP are quite similar, as can be seen in Figure 2A and Table 5. Figure 2B shows that the ADP binding kinetics are different, as expected from the data obtained for ATP as a competitor of mant-GDP binding. Whereas the plot showing the pseudo-first-order rate constant for GDP or IDP association with the Y32W mutant is hyperbolic starting from zero at low nucleotide concentrations, the plot with ADP, while hyperbolic, obviously does not start from zero at low ADP concentrations, and the whole curve is shifted toward much higher nucleotide concentrations. The results are consistent with the same two-step binding scheme as was used to explain the results obtained from fluorescent nucleotides with wild-type p21 or from GDP or IDP with the the Y32W mutant, with the difference that the reverse rate constant for the second step is not now immeasurably small on the time scale of the experiment but has a measurable value which is given by the intercept of the hyperbola with the ordinate. This behavior is described by the following equation, which can be derived under the assumption that the first step is in rapid equibrium in comparison with the second step (more rigorously, under the assumption that k_{-1} is much larger than $k_{+2} + k_{-2}$, which seems reasonable in view of the very weak association in the first step):

$$k_{obs} = k_{+2}/(1 + 1/K_1[N]) + k_{-2}$$

where the rate constants are as defined in Scheme 1 and k_{obs} is the pseudo-first-order rate constant for the fluorescence change seen in the stopped-flow experiment.

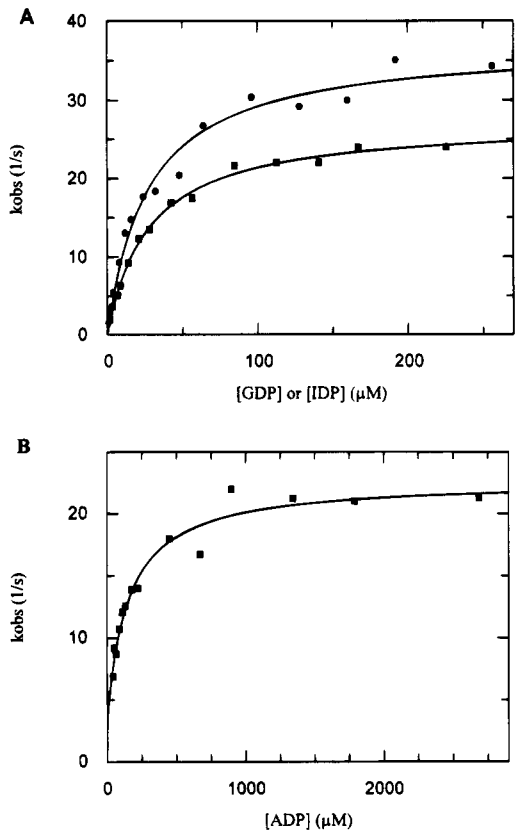

FIGURE 2: (A) Dependence of the pseudo-first-order rate constant for the association of GDP (●) or IDP (■) with 0.5 μM p21 Y32W on the concentration of nucleotide. Data were collected using a stopped-flow apparatus with trypophan fluorescence as a signal of binding. Individual curves could be fitted with a signal-exponential term. (B) As in (A), but using ADP. Conditions as in Figure 1.

Table 5: Kinetics of Interaction of GDP, IDP, and ADP with the Y32W Mutant of p21[a]

nucleotide	K_1 (M^{-1})	k_{+2} (s^{-1})	k_{-2} (s^{-1})
GDP	4×10^4	36	$<10^{-4}$
IDP	3.7×10^4	27	<0.1
ADP	6.6×10^3	19	3.3

[a] K_1 and k_{+2} were determined as described in the text using the stopped-flow method a [and tryptophan fluorescence as a signal of binding (data shown in Figure 2)]. In the case of ADP, the measurable intercept on the y-axis allows determination of k_{-2} from the data of Figure 2B. k_{-2} was not determined directly for GDP, but k_{-2} for the more weakly bound mant-dGDP was found to be 0.8×10^{-4} s^{-1} (1×10^{-3} s^{-1} for wild-type p21). k_{-2} for IDP was also not determined directly, and the upper limit of 0.1 s^{-1} obtained from Figure 2 is probably at least an order of magnitude higher than the real value.

The values obtained for K_1, k_{+2}, and k_{-2} in the case of ADP are similar to those obtained for ATP in the competition experiment with mant-GDP, in agreement with the observation that nucleoside triphosphates are bound with similar affinity and kinetic constants to those of diphosphates.

Measurement of the rates of dissociation of ITP and XTP from their complexes with p21 led to the values shown in Table 4. It can be seen that these are about 2 orders of magnitude higher than for mant-GDP (or GDP or GTP) and that this is the main source of the lower affinity of the nucleotides for p21. There appears to be little discrimination against ITP or XTP in the initial binding. In contrast, ATP binds about 2 orders of magnitude less strongly in the first step. There is even more significant discrimination in the second step. For GDP and GTP, this has an equilibrium constant of ca. 10^6. This drops to ca. 10^4 for ITP and XTP and, most dramatically, to 10 for ATP. The kinetics of GMP and guanosine binding to p21 suggest that the value is even lower for these nucleotides, and that this leads to essentially single-step binding in this case. For this reason, the overall affinity of p21 for GMP and guanosine has been equated with K_1 in Scheme 1.

NTPase Activity of p21. Since ITP and ATP interact with p21 in a manner which is similar in terms of the kinetic mechanism to the interaction of GTP, it is of interest to examine their properties as substrates. The Y32W mutant provides an easy way of doing this, since cleavage can be followed using the fluorescence signal as a monitor of the state of the nucleotide at the active site. With ITP, a similar result was obtained as with GTP; i.e., there was a slow increase in tryptophan fluorescence after binding of ITP to the nucleotide-free mutant form of the enzyme. The rate constant for this process was 1.1×10^{-4} s^{-1}, which is similar to the value obtained for GTP with this mutant (1.4×10^{-4} s^{-1}). This could be accelerated dramatically by addition of GAP, which suggests that the inosine triphosphatase mechanism of p21 and GAP−p21 is the same as for the GTPase mechanism. With ATP, it was not possible to show unequivocally whether it is a substrate or not. This is because of the weak interaction of p21 with ATP, and because the nucleotide-free protein contains traces of alkaline phosphatase actvity which arise from the procedure used for removal of nucleotide (John et al., 1990). This is not a problem with nucleotides which bind tightly to p21, since a 1:1 complex can be generated which dissociates so slowly that the nucleotide is not available to alkaline phosphatase in the solution. This does not apply to the weakly bound ATP, and degradation to adenosine occurred quite rapidly in attempts to measure the ATPase activity of p21. No acceleration of ATP degradation was seen in the presence of GAP, so that we conclude tentatively that ATP is not a substrate for p21.

The Mechanism of Base Discrimination by p21. The kinetic results summarized in the tables allow certain interpretations to be made about the nature of the interactions occurring at each of the steps in the binding mechanism. K_1 appears to be of similar magnitude for GTP, GDP, GMP, guanosine, ITP, and XTP, but much lower for ATP. On inspecting the structures of these ligands, it is seen that the structural feature which the first six have in common, and which is different for ATP, is the structure around the N-1 and C-6 positions of the purine base. It therefore seems likely that interactions between the protein and this part of the base structure are important for the first step. Neither of the two interactions which can be seen in the high-resolution p21·GppNHp structure (the NH group at position 1 of the base with the carboxyl group of Asp-119 and the carbonyl group at position 6 with the backbone NH of Ala-146) would be possible with adenine as the base moiety. However, it seems unlikely that the interaction of Asp-119 with N-1 would occur without the simultaneous interaction (via the second oxygen of the carboxyl group) with the NH_2 group at position 2, and this should lead to discrimination against ITP and XTP. Since this discrimination is not apparent, we conclude that there is probably only a strong interaction with the oxygen at position 6 in the first bound state of the nucleotides (possibly in addition to other

interactions which will not discriminate between different purine bases, e.g., the interaction of Asn-116 with N7 and the aromatic interaction of the side group of Phe-28 with the aromatic ring system).

Discrimination against ITP and XTP occurs mainly in the second step. Interestingly, the rate of the forward reaction appears to be independent of the nature of the nucleotide, since it is the same for GTP, GDP, ITP, XTP, and ATP. Differences between these nucleotides are solely in the reverse rate constants, which vary over 5 orders of magnitude. A possible interpretation of this behavior is that the rate of the forward reaction is that of a protein structural change which can occur regardless of the presence or nature of the nucleotide at the active site. The equilibrium constant for this reaction is governed by the magnitude of the reverse reaction, which is, presumably, large (i.e., much larger than 22 s^{-1}) in the absence of nucleotide or when GMP or guanosine is at the active site, but very small when GDP or GTP is bound. Since there is discrimination against the base and against the "wrong" number of phosphate groups (zero or one as opposed to two or three), we must assume that important interactions of both the base and phosphate moieties with the protein are established in this reaction. It seems likely that the important interaction with the base which is formed in this step is that between Asp-119 and N-1 and and N-2. As seen from the high-resolution p21•GppNHp and p21•GDP structures, there are a large number of important interactions with the β- and γ-phosphate groups. Some of these interactions also involve the Mg^{2+} ion, which interacts with the β- and γ- phosphate groups on the one hand, and Ser-17 and Thr-35 on the other hand. These interactions would not be possible with GMP or guanosine at the active site.

The nucleotide-binding properties of position 119 mutants have been investigated (Sigal et al., 1986a,b; Feig et al., 1986), and it has been shown that replacement with a residue lacking the carboxyl group of the aspartate (e.g., alanine) leads to higher dissociation rates of guanosine nucleotides and a reduction of the discrimination against IDP from a factor of 100 for wild-type protein to a factor of 10 for the mutant.

We were particularly intrigued by the dramatic discrimination of p21 between GTP and ATP. From the high-resolution structure of p21•GppNHp (Pai et al., 1990), it is apparent that three of the hydrogen bonding interactions between the protein and guanine are not possible in the case of adenine (the Asp-119 side chain to N1 and N2, and the backbone NH of Ala-146 to O6). While these are obviously important interactions, it does not seem reasonable that the loss of them should result in a loss of affinity by a factor of almost 10^7. An estimate of the contribution of these three interactions to the overall affinity comes from the relative affinity of the triphosphate of purine riboside, which is bound a factor of 10^3 less strongly than GTP. It thus seems that in addition to the loss of binding energy contributed by these interactions, there must be some unfavorable interactions in the case of ATP. The nature of this unfavorable interaction was evident from a computer experiment in which GTP was replaced by ATP at the active site of p21. This leads to an impossibly close contact between the hydrogen atoms of the exocyclic amino group of ATP and the backbone NH of Ala-146. On allowing the program XPLOR to search for the structural arrangement with the lowest energy, it is seen that neither the polypeptide backbone around residue 146 nor the rest of the nucleotide can be positioned so as to avoid this unfavorable interaction. The explanation for the discrimination against ATP thus appears to be that there is steric hindrance caused by the exocyclic amino group. The fact that this negative interaction occurs with the backbone of the protein means that the ideas and conclusions obtained here cannot be easily tested by site-directed mutagenesis with the aim of changing the specificity from GTP to ATP. However, preliminary computer modeling experiments suggest that exchanging several amino acids in the region of residue 146 might change the position of the backbone sufficiently to allow the negative interaction to be removed. A promising approach to this question might be to combine random mutagenesis of a number of residues around Ala-146 (in conjunction with a selection method which would select for increased ATP or ADP affinity relative to that of GTP or GDP) with site-specific mutagenesis to replace side chains by groups which could change specificity because of their interactions with other parts of the nucleobase, in particular at position 119.

It is of interest to consider the significance of the high specificity of guanosine nucleotide binding to p21 and other related proteins involved in signal transduction or in protein transport or any other process in which GTP/GDP-binding proteins are involved. While it is possible that the selection of GTP/GDP binding as opposed to ATP/ADP was a chance event in evolution, it is conceivable that there are good mechanistic or physiological reasons for this to be the better choice. One factor which may be of importance is that ATP is involved in a large number of energy-transducing processes which result in the turnover of substantial amounts of ATP. Although enzymes involved in nucleotide metabolism tend to buffer the concentration variations arising from such processes, it seems likely that there are larger temporal variations in the ATP/ADP ratio than in the GTP/GDP ratio in the cell. Since the rate constants of association of GTP and GDP are approximately equal, at least in the case of p21, the relative concentrations of p21•GTP and p21•GDP which are produced after release of GDP is catalyzed by an exchange factor are determined solely by the relative concentrations of GTP and GDP. While rebinding of GDP would lead to repeated release in the presence of an active exchange factor, varying concentration relationships of the nucleotide would lead to varying response to a signal, which might not be desirable.

A mechanistic reason for the choice of GTP could be connected with the fact that the role of the guanosine nucleotide-binding proteins in signal transduction and other related processes requires a high intrinsic affinity between the nucleotides and the proteins, this affinity being modulated by exchange factors. The chemical nature of the guanine base could offer more "opportunities" for interactions with the protein, or perhaps a better starting point for highly specific interactions. It is known that some enzymes have a very high affinity for ATP, an example being myosin, with a K_a value of ca. 10^{11} M^{-1} (Mannherz et al., 1974; Goody et al., 1976). However, much of the affinity in this case arises from interactions with the γ-phosphate group, since the association constant drops to 10^6 M^{-1} for ADP, and the base specificity is much less pronounced than for p21. In the case of p21, GTP and GDP have similar affinity, suggesting that more of the ineraction energy comes from the inter-

actions of the base with the protein than in the case of myosin. Further observations in keeping with this analyisis are that inorganic pyrophosphate binds with relatively high affinity to the β,γ-phosphate-binding sites of myosin and can mimic ATP in dissociating the stable actomyosin complex, whereas there is no detectable interaction between pyrophosphate and p21 (H. Rensland and R. S. Goody, unpublished results).

The results described allow a number of interpretations to be made concerning events occurring in the two steps in the nucleotide-binding process. It seems likely that p21•GMP and p21•guanosine are analogs of the first bound state seen with GDP and GTP, since the kinetic characteristics are similar. Discrimination against ATP occurs at this stage, as shown by the lack of interaction of AMP with p21 (data not shown) and the hundredfold lower equilibrium constant for the first step in the case of ATP when compared with GTP or GDP. Since the second step occurs with GTP, GDP, and even ATP, but not with GMP or guanosine, it seems likely that this step involves formation of the strong interaction between the active site of the enzyme and the β-phosphate group, as well as establishing specific interactions with the base, as discussed before. In a hypothetical model of the binding process, the first step would lead to some or all of the interactions between the base and its binding pocket. Since the rate of the second step is similar for GDP, GTP, or ATP, we suggest that this involves, as the rate-limiting step in a process which could perhaps be broken down into further steps, an isomerization of the protein, in particular of the active site, which is an inherent property of the protein and which is not influenced directly by the bound nucleotide. This results in docking of the phosphate groups onto their binding sites. In ATP, the poor positioning of the base in its binding site would lead to poor interaction of the phosphates with their binding sites.

It would be of interest to characterize the conformational change associated with the second step in the binding mechanism. Apart from the general interest in the nature of processes involved in substrate-induced structural changes in enzymes, it is possible that a knowledge of the structural change would shed light on the mechanism of the acceleration of the GDP/GTP exchange reaction by exchange factors. An attractive possibility is that the exchange factor binds to p21 and alters (decreases) the value of the equilibrium constant K_2 in Scheme 1, thus effectively increasing the rate of dissociation because of the rapid dissociation of nucleotide from the first bound state in the binding mechanism. Information on the first bound state of the nucleotide might be obtained from determination of the structure of p21 with GMP or guanosine at the active site, since the results obtained in the present work suggest that the second step in the binding mechanism does not occur with these ligands. The complex between p21 and guanosine has been crystallized, and work toward determination of its structure is presently in progress (Scheffzek et al., 1994).

ACKNOWLEDGMENT

We thank Marija Matuska for synthesis of nucleotides and Kenneth C. Holmes for continued support.

REFERENCES

deVos, A. M., Tong, L., Milburn, M. V., Matias, P. M., Jancarik, J., Noguchi, S., Nishimura, S., Miura, K., Ohtsuka, E., & Kim, S.-H. (1988) *Science 239*, 888−893.

Feig, L. A., Pan, B.-T., Roberts, T. M., & Cooper, G. M. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 4607−4611.

Feuerstein, J., Kalbitzer, H. R., John, J., Goody, R. S., & Wittinghofer, A. (1987) *Eur. J. Biochem 162*, 49−55.

Goody, R. S., & Isakov, M. (1986) *Tetrahedron Lett. 27*, 3599−3602.

Goody, R. S., Hofmann, W., & Mannherz, H. G. (1977) *Eur. J. Biochem. 78*, 317−324.

Goody, R. S., Frech, M, & Wittinghofer, A (1991) *Trends Biochem. Sci. 16*, 327−328.

Goody, R. S., Pai, E. F., Schlichting, I., Rensland, H., Scheidig, A., Franken, S., & Wittinghofer, A. (1992) *Philos. Trans. R. Soc. London, B. 336*, 3−11.

John, J., Frech, M., Feuerstein, J., Goody, R. S., & Wittinghofer, A. (1989) in *Guanine-Nucleotide Binding Proteins*, pp 209−214, Plenum Press, New York.

John, J., Sohmen, R., Feuerstein, J., Linke, R., Wittinghofer, A., & Goody, R. S. (1990) *Biochemistry 29*, 6059−6065.

Mannherz, H. G., Schenck, H., & Goody, R. S. (1974) *Eur. J. Biochem. 48*, 287−295.

Milburn, M. V., Tong, L., DeVos, A. M., Brünger, A., Yamaizumi, Z., Nishimura, S., & Kim, S-H. (1990) *Science 247*, 939−945.

Neal, S. E., Eccleston, J. F, & Webb, M. R. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 3652−3565.

Noonan, T., Brown, N., Dudyc, L., & Wright, G. (1991) *J. Med. Chem. 34*, 1302−1307.

Pai, E. F., Kabsch, W., Krengel, U., Holmes, K. C., John, J., & Wittinghofer, A. (1989) *Nature 341*, 209−214.

Pai, E. F., Krengel, U., Petsko, G. A., Goody, R. S., Kabsch, W., & Wittinghofer, A. (1990) *EMBO J. 9*, 2351−2359.

Rensland, H., Lautwein, A., Wittinghofer, A., & Goody, R. S. (1991) *Biochemistry 30*, 11181−11185.

Scheffzek, K., Kabsch, W., Schlichting, I., Pai, E. F., Lautwein, A., Frech, M., Wittinghofer, A., & Goody, R. S. (1994) *Acta Crystallogr. D50*, 521−526.

Schlichting, I., Almo, S. C., Rapp, G., Wilson, K., Petratos, K., Lentfer, A., Wittinghofer, A., Kabsch, W., Pai, E. F., Petsko, G. A., & Goody, R. S. (1990) *Nature 345*, 309−315.

Sigal, I. S., Gibbs, J. B., DÁlonzo, J. D., & Scolnick, E. M. (1986a) *Proc. Natl. Acad. Sci. U.S.A. 83*, 4725−4729.

Sigal, I. S., Gibbs, J. B., DÁlonzo, J. D., Temeles, G. L., Wolanski, B. S., Socher, S. H., & Scolnick, E. M. (1986b) *Proc. Natl. Acad. Sci. U.S.A. 83*, 952−956.

Tucker, J., Sczakiel, G., Feuerstein, J., John, J., Goody, R. S., & Wittinghofer, A. (1986) *EMBO J. 5*, 1351−1358.

BI9420626