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Biochemistry

Reversal of Rous sarcoma-specific immunoglobulin phosphorylation on tyrosine (ADP as phosphate acceptor) catalyzed by the *src* gene kinase

(tumor-bearing rabbit serum/protein-bound tyrosine *O*-phosphate–ADP equilibrium constant/$\Delta G^{\circ\prime}$ of hydrolysis)

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ABSTRACT **To determine the equilibrium constant of the reaction between ATP and protein-bound tyrosine we used as catalyst the highly purified Rous sarcoma *src* gene transcript. J. M. Sturtevant had earlier found (personal communication) that free tyrosine *O*-phosphate, upon hydrolysis with alkaline phosphatase in a calorimeter (37°C, pH 9), yielded a ΔH° of −2.8 kcal/mol (1 kcal = 4.18 kJ), less than half of that found in ATP hydrolysis. Experience with protein-bound serine phosphate (in phosvitin) had shown it to be energy rich [Rabinowitz, M. & Lipmann, F. (1960) *J. Biol. Chem.* 235, 1043–1050]. We wondered if the same is true for tyrosine phosphate when it is protein bound. From the equilibrium constant of 2.62 (at pH 6.5 and 5 mM Mg^{2+}), we calculate a $\Delta G^{\circ\prime}$ of −9.48 kcal/mol for hydrolysis of protein-bound tyrosine phosphate, assuming an approximate $\Delta G^{\circ\prime}$ of −10 kcal/mol for hydrolysis of ATP. The experiments show that protein-bound tyrosine phosphate is energy rich, like serine phosphate in phosvitin.**

Erikson *et al.* (1) discovered that the translation product of the transforming gene of Rous sarcoma virus (RSV) is a twice phosphorylated 60,000 M_r protein ($pp60^{src}$), and it was found to be a protein kinase. This observation connected a phosphate transfer to proteins with a malignant transformation. An important feature of this kinase—namely, specifically phosphorylating the hydroxyl group of tyrosine—was found by Hunter and Sefton (2).

The $pp60^{src}$ kinase is itself phosphorylated on a tyrosine near the COOH terminus and, through a cyclic-AMP-linked enzyme, also on a serine near the NH_2 terminus. The tyrosine-specific phosphorylation of IgG from RSV-tumor-bearing rabbit (TBR) was shown by Erikson *et al.* (1) in a temperature-sensitive strain of RSV to be deficient at the nonpermissive temperature and thus connected with the activity of $pp60^{src}$. The transfer of the γ-*P* of ATP to the specific immunoprotein had earlier been observed to occur by the interaction between TBR serum IgG and $pp60^{src}$ + [γ-^{32}P]ATP. Also, a 34,000 M_r protein was found by Radke and Martin (3) to accept [^{32}P]*P* on tyrosine in chicken embryo fibroblasts (CEF) infected with a temperature-sensitive strain of RSV but only after transition from nonpermissive to permissive temperature. Note that in both cases the site of phosphorylation was a tyrosine (4). The level of tyrosine *O*-phosphate (Tyr-*P*) in normal tissue appears to be very low, but it increases 5- to 10-fold in CEF after infection with RSV. Until recently, an adenylylation on tyrosine in *Escherichia coli* glutamine synthetase had been observed by Stadtman (5), but no incorporation of *P* into protein-bound tyrosine had been known.

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Nitrophenyl phosphate esters are known to carry energy-rich phosphates. To a first approximation, their heat of hydrolysis (catalyzed by phosphatase) was determined by Sturtevant (6), and it was found to be similar to the ΔH° of hydrolysis of γ-*P* in ATP. It is not possible, however, to generalize a high phosphate potential to all phenolic phosphates, as, for example, Tyr-*P*. Indeed, in a preceding paper (7) on the free Tyr-*P* in *Drosophila* larvae (8) we found only a slightly reversible tyrosine-specific phosphatase and no kinase or protein-bound Tyr-*P*. We suspected then that the *P* potential in Tyr-*P* was not very high. Earlier work here with Rabinowitz (9) had shown, however, that, in contrast to free serine *O*-phosphate (Ser-*P*), the Ser-*P* of [^{32}P]phosvitin in the presence of a kinase transfers [^{32}P]*P* to ADP to form [γ-^{32}P]ATP and, thus, in the protein it has become energy rich.

Purified Tyr-*P* had been made during the earlier work (7), and we offered this to J. M. Sturtevant of Yale University. He was interested in a determination of its heat of hydrolysis, ΔH°, with the microcalorimeter in his laboratory. With alkaline phosphatase catalyzing the hydrolysis, the results of such experiments by J. M. Sturtevant and Richard Epand of McMaster University yielded a ΔH° of −2.8 kcal/mol (1 kcal = 4.18 kJ), which is considerably below that of nitrophenyl phosphate or γ-*P* of ATP determined earlier in the Sturtevant laboratory (6).

In this paper TBR-IgG will be used as the protein that accepts phosphate on its tyrosine. The site of the specifically phosphorylated tyrosine in TBR-IgG is in the heavy chains (10).

Our finding of an easily reversible reaction between Tyr-*P* in the TBR-IgG and ADP, with $pp60^{src}$ as catalyst, encouraged us to use this system for the evaluation of the equilibrium constant between ATP and the TBR-IgG Tyr-*P* for determining the free energy (ΔG°) of hydrolysis of protein-bound Tyr-*P*. It will be shown that we did succeed in determining this equilibrium constant. The result indicates that the phosphate potential in TBR-IgG Tyr-*P* is approximately on the level of the γ-*P* in ATP.

EXPERIMENTAL PROCEDURES

Materials. TBR serum prepared according to ref. 11 was generously provided by R. Feldman and H. Hanafusa (The Rockefeller University), and the same batch was used throughout the experiments. Normal rabbit serum was purchased from

Abbreviations: RSV, Rous sarcoma virus; $pp60^{src}$, phosphorylated protein product of *src* gene; TBR, tumor-bearing rabbit; Tyr-*P*, tyrosine *O*-phosphate; Ser-*P*, serine *O*-phosphate; Thr-*P*, threonine *O*-phosphate; CEF, chicken embryo fibroblasts.

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Flow Laboratories. L-Tyr-*P* was synthesized and purified as described (7) according to the method of Mitchell and Lunan (8). Ser-*P*, threonine phosphate (Thr-*P*), ATP, ADP, GDP, and hexokinase were obtained from Sigma. ATP, ADP, and GDP were purified by DEAE-Sephadex column chromatography (12). [γ-^{32}P]ATP (5,000 Ci/mmol; 1 Ci = 3.7×10^{10} Bq) was obtained from Amersham.

Preparation of RSV *src* Gene Kinase. The *src* gene kinase was purified from CEF transformed by RSV (Schmidt–Ruppin A strain). The cultured RSV-transformed cells were extracted with 0.5% Triton X-100-containing buffer A [25 mM Tris·HCl, pH 7.5/1 mM EDTA/1 mM dithiothreitol/0.2 mM phenylmethylsufonyl fluoride/10% (vol/vol) glycerol] supplemented with aprotinin (Boehringer Mannheim), a trypsin inhibitor, at 1 unit/ml. The extract was cleared by centrifugation (100,000 × *g*, 30 min) and the supernatant fraction was applied to a casein-agarose column (United States Biochemical, Cleveland, OH; 1.5 × 28 cm). After the column had been washed with buffer B (buffer A containing 0.1% Triton X-100), the *src* gene kinase was eluted by a linear gradient of NaCl in buffer B. The peak fractions were pooled, adjusted to 0.8 M $(NH_4)_2SO_4$, and applied to an L-tyrosine-agarose column (Sigma, 1.5 × 18 cm) equilibrated with 0.8 M $(NH_4)_2SO_4$ in buffer B. Elution was done by decreasing the concentration of $(NH_4)_2SO_4$. The eluted enzyme was dialyzed against buffer B and applied at low ionic strength to a second tyrosine-agarose column, which had been equilibrated with buffer B. It was then eluted by increasing concentrations of $(NH_4)_2SO_4$ in buffer B. The final fraction was found to be purified about 7,800-fold compared to the cleared supernatant. The method will be described in detail in a separate publication.

Phosphorylation of TBR-IgG. Standard reaction mixtures (25 μl) contained 50 mM Tris·HCl at pH 7.5, 5 mM $MgCl_2$, 1 mM dithiothreitol, 2 μM [γ-^{32}P]ATP (500 Ci/mmol), 5 μl of TBR serum diluted 1:50, and the purified *src* gene kinase. The reaction was carried out at 30°C for 20 min unless otherwise indicated (as it is in the legends to Fig. 4 and Table 1) and was terminated by adding 5 μl of 0.1 M EDTA to chelate the Mg^{2+}. IgG was adsorbed onto protein A-containing *Staphylococcus aureus* (Pansorbin, Calbiochem-Behring) at 7 μl of 10% suspension per standard reaction mixture and was washed three times with buffer B. The immune complex was suspended in 25 μl of sample buffer (0.025 M Tris·HCl (pH 6.8)/2% $NaDodSO_4$/10% glycerol/5% 2-mercaptoethanol/0.001% bromophenol blue) and heated at 100°C for 3 min. The bacteria were pelleted by centrifugation, and the supernatant was analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis according to the method of Laemmli (13), using 5.5–15% acrylamide gradient gels. Gels were stained with 0.25% Coomassie brilliant blue in water/methanol/acetic acid, 55:35:10 (vol/vol), and destained in the same solution without the dye, dried, and autoradiographed. IgG bands were cut out and their radioactivities were measured in a liquid scintillation counter with 5 ml of Hydrofluor (National Diagnostics). One unit of kinase was defined as the amount of enzyme that in 20 min at 30°C catalyzes transfer of 10 fmol of [^{32}P]*P* from [γ-^{32}P]ATP to TBR-IgG with the formation of ADP. Phosphorylation by *src* gene phosphorylase occurred only on TBR-IgG and not on normal IgG (Fig. 1*A*) and the amino acid phosphorylated was exclusively tyrosine (Fig. 1*B*).

Reverse Transfer from ^{32}P-Phosphorylated IgG to ADP. The mixture for the reverse reaction contained 50 mM Tris·HCl at pH 7.5, 5 mM $MgCl_2$, 1 mM dithiothreitol, various amounts of ADP, 10 μl of phosphorylated IgG–Pansorbin suspension (9,000–20,000 cpm), and 2.5 units of *src* gene kinase. Phosphorylated IgG adsorbed on Pansorbin was prepared as described in the above section, except that before the addition of Pansorbin the reaction product was passed through a gel filtration column of Sephadex G-75 (0.5 × 8 cm) to remove free ATP. The reverse reaction mixture was incubated at 30°C for 20 min, then the IgG-[^{32}P]*P*–Pansorbin complex was pelleted by centrifugation. An aliquot of 10 μl of the supernatant was analyzed for ATP formation by polyethyleneimine (PEI)-cellulose (Nagel) thin-layer chromatography with 0.5 M KH_2PO_4 at pH 3.5 (14) and the pellet fraction was subjected to $NaDodSO_4$/polyacrylamide gel electrophoresis to measure the [^{32}P]ATP formed by the dephosphorylation of IgG-[^{32}P]*P*.

RESULTS

Tyrosine-Specific Phosphorylation of TBR-IgG with [γ-^{32}P]ATP and Reversal from IgG-[^{32}P]*P* to ADP. IgG was phosphorylated by the *src* gene kinase with [γ-^{32}P]ATP and the product was analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis (Fig. 1*A*). The radioactivities are represented in Fig. 2*B*.

The reverse reaction—i.e., phosphate transfer from phosphorylated IgG to ADP—is demonstrated in Fig. 2. Formation of radioactive ATP depended on the concentration of added ADP, and concomitantly, the radioactivity on IgG decreased (lanes 1–7). Because aliquots of IgG-[^{32}P]*P* were used in the reversal of phosphorylation to form [^{32}P]ATP the spots on the upper line for [γ-^{32}P]ATP recovered by addition of increasing amounts of ADP represent relative and not absolute values. Like phosphorylation (15), the dephosphorylation was dependent on addition of magnesium (lane 8) or manganese (not shown). Addition of GDP in place of ADP resulted in the somewhat lesser formation of GTP (lane 9).

Stoichiometry of the phosphate transfer is shown in Fig. 3. Formation of ATP was approximately complementary to dephosphorylation of IgG, and practically no loss of radioactivity was observed. It is also clear from Fig. 2 that no protein phosphatase or ATPase was contaminating the reaction system.

Determination of the Equilibrium Constant. The maximal level of IgG phosphorylation by saturation with enzyme was

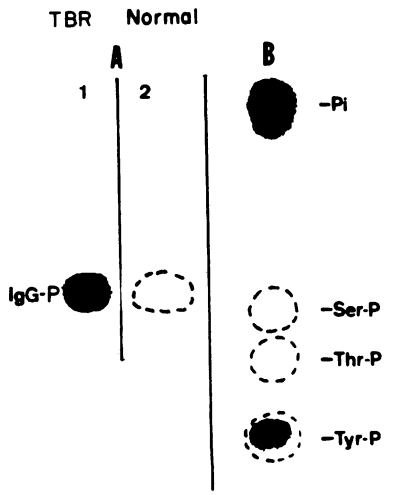

FIG. 1. Phosphorylation of TBR-IgG and phospho amino acid analysis. (*A*) The phosphorylation of IgG was assayed in TBR serum (lane 1) and normal rabbit serum (lane 2). The products were analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis and autoradiography. (*B*) For analysis of phosphorylated amino acids, the phosphorylated TBR-IgG was extracted from the gel in *A* and hydrolyzed in 6 M HCl at 110°C for 2 hr. The hydrolysate was subjected to cellulose thin-layer electrophoresis at pH 3.5 as described by Hunter and Sefton (2) with nonradioactive standards that were localized by ninhydrin staining (shown by the dashed circles).

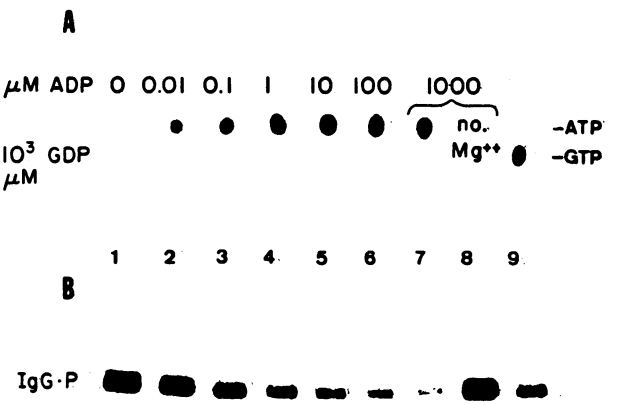

FIG. 2. Reverse phosphate transfer from phosphorylated IgG to ADP. Phosphorylated IgG adsorbed on protein A-containing *Staphylococcus aureus* was prepared and incubated in the reverse reaction mixture. After 20 min of incubation at 30°C, the immune complex was pelleted by centrifugation. An aliquot (10 μl) of the supernatant was analyzed by PEI-cellulose thin-layer chromatography (*A*), and the pellet fraction was analyzed for dephosphorylation of IgG (*B*) by $NaDodSO_4$/polyacrylamide gel electrophoresis. Reactions were performed with the indicated additions: Lanes 1–7, 0, 0.01, 0.1, 1.0, 10, 100, and 1,000 μM ADP, respectively; lane 8, 1 mM ADP without $MgCl_2$; lane 9, 1 mM GDP.

determined as shown in Fig. 4. In the inset of Fig. 4, the saturation of IgG is measured with increasing amounts of [γ-^{32}P]ATP and the enzyme in the saturating concentration. The maximal amount of phosphate incorporated was 15.42 fmol per 25 μl of the standard reaction mixture, corresponding to 617 pM reactive sites on IgG. This value was used to calculate the total amount of TBR-IgG involved in the phosphorylation by the *src* gene kinase. The phosphorylation reaction with constant [γ-^{32}P]ATP can be written as follows:

$$\text{IgG} + \text{ATP} \rightleftharpoons \text{IgG-}P + \text{ADP}. \qquad [1]$$

The justification of this assumption is proven experimentally by obtaining approximately equal *K* values independent of the ratios of ADP to ATP as shown in Table 1. The equilibrium

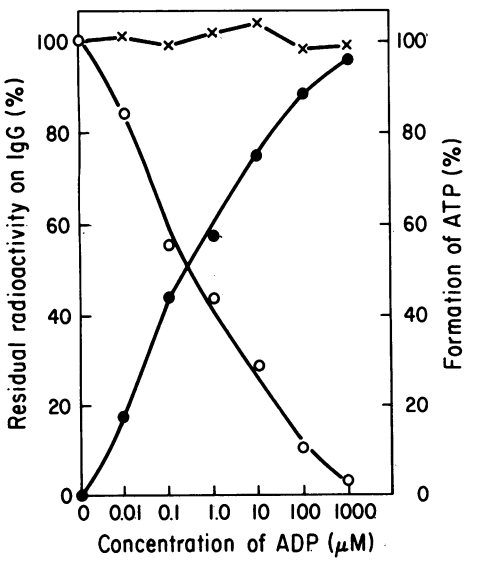

FIG. 3. Stoichiometry of phosphate transfer. Reverse reactions were carried out and the radioactivities remaining on IgG and those recovered in ATP were plotted against the concentrations of ADP used in the reverse reactions. The initial radioactivity (9,200 cpm) on the IgG heavy chain was taken as 100%. ○, Residual radioactivity on IgG; ●, radioactivity recovered in ATP; ×, total radioactivity (○ + ●).

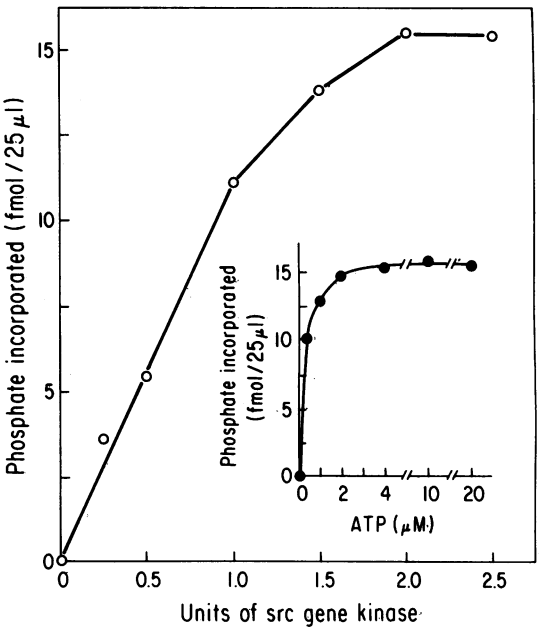

FIG. 4. Maximal phosphorylation of IgG. Phosphorylation was performed in the standard reaction mixture with various amounts of enzyme at the saturating ATP concentration of 20 μM. Insert: The phosphorylation here was carried out at various concentrations of ATP with the saturating amount of 2.5 units of enzyme. In both cases, the incubation time was 45 min instead of the 20 min in the standard procedure.

constant K_{eq} is calculated by using

$$K_{eq} = \frac{[\text{IgG-}P]}{[\text{IgG}]} \times \frac{[\text{ADP}]}{[\text{ATP}]}. \qquad [2]$$

The total RSV-specific IgG can be set as corresponding to the maximal amount of phosphate incorporated: 15.42 fmol/25 μl = 617 pM reactive IgG.

The reproducibility of the reverse reaction of IgG-[^{32}P]*P* + ADP carried out in the suspension of the IgG–Pansorbin complex proved to be unsatisfactory, with more than 30% deviation, and our sera showed no detectable phosphatase or ATPase activity. Therefore, in the equilibrium experiments, free IgG was used as substrate. The experiments in Table 1 show that the partial phosphorylation of IgG depends reliably on the various ratios of ADP to [γ-^{32}P]ATP. However, to measure the amount of phosphorylated IgG after the equilibrium was established, Pansorbin had to be used to collect the IgG-[^{32}P]-*P*; then excess [γ-^{32}P]ATP was removed as described.

The concentration of free IgG in Eq. 2 is calculated by subtraction of the pM IgG-[^{32}P]*P* from 617 pM total IgG. The ratio of ADP to ATP, depending only on the variable concentrations of ADP, can be considered as remaining constant during the equilibrium reaction, in view of the negligible amount of ^{32}P transferred to IgG as compared with the constant amount of [γ-^{32}P]ATP added (see legend of Table 1).

Calculation of the Free Energy of Hydrolysis of IgG-*P* from the Equilibrium Constant. We are greatly indebted to John D. Gregory for his help with this evaluation. The first step is the calculation of the free energy $\Delta G^{\circ\prime}$ from the equilibrium constant, K_{eq}, as listed in the last column of Table 1:

$$\Delta G^{\circ\prime} = RT \ln K_{eq}$$

$$\Delta G^{\circ\prime} = 1.4 \log K_{eq} \text{ (kcal)},$$

in which *R* is the gas constant and *T* is the absolute temperature.

Table 1. Apparent equilibrium constants

Exp.	pH	$MgCl_2$, mM	ADP, μM	[^{32}P]ATP, μM	IgG-*P*, pM	[IgG-*P*]/[IgG]		K_{eq}
1	7.5	5	1	2.0	571	12.4		6.20
2	7.5	5	2	2.0	550	8.21		8.21
3	7.5	5	4	2.0	475	3.28		6.56
4	7.5	5	10	2.0	355	1.35		6.75
							Mean	6.93
5	7.5	10	1	2.0	596	28.4		14.2
6	7.5	10	2	2.0	562	10.2		10.2
7	7.5	10	10	2.0	420	2.13		10.7
							Mean	11.7
8	6.5	5	1	2.0	496	4.10		2.05
9	6.5	5	10	2.0	214	0.531		2.66
							Mean	2.36
10	6.5	10	1	2.0	548	7.94		3.97
11	6.5	10	10	2.0	271	0.783		3.92
							Mean	3.95
12	8.0	5	1	2.0	584	17.7		8.85
13	8.0	5	10	2.0	336	1.20		6.00
							Mean	7.43
14	8.0	10	10	2.0	436	2.41		12.1

Equilibrium reactions were carried out at various ADP concentrations with a constant [γ-^{32}P]ATP concentration in the standard reaction mixtures (25 μl) containing 50 mM Tris·HCl at pH 7.5, 5 mM $MgCl_2$, 1 mM dithiothreitol, 5 μl of TBR serum diluted 1:50, and 2.5 units of the *src* gene kinase. For experiments at pH 6.5, 50 mM Tris acetate buffer was used instead of Tris·HCl. Reaction mixtures were incubated at 30°C for 45 min, and the equilibration with the different ratios of ADP to [γ-^{32}P]ATP was done with free IgG. To collect the IgG-[^{32}P]*P*, the Pansorbin complex had to be used as outlined in the text. The added concentrations of ATP and ADP were taken as the final concentrations, because the concentration of *P* transferred to IgG was very small compared to the concentrations of ATP and ADP and the concentration change of ATP and ADP during the reaction was negligible (less than 0.06%). The equilibrium constant with variation of the quotient [ADP]/[ATP] was calculated by the equation

$$K_{eq} = \frac{[\text{IgG-}P]}{[\text{IgG}]} \times \frac{[\text{ADP}]}{[\text{ATP}]}.$$

The quotient [ADP]/[ATP] on the right remains as mentioned, essentially equal to the quotient of the added amounts; the quotient [IgG-*P*]/[IgG] is calculated from the measured radioactivity of IgG-[^{32}P]*P*. The free IgG is obtained in pM by subtracting the IgG-[^{32}P]*P* from 617 pM total IgG, which was determined by maximum [^{32}P]*P* incorporation with saturating amounts of [γ-^{32}P]ATP and the kinase (Fig. 4).

Using the average value K_{eq} of 2.36 for the optimal pH of 6.5 and Mg^{2+} of 5 mM, we obtain $1.4 \times 0.372 = 0.52$ kcal/mol.

We add now the reversed Eq. 1 for $\Delta G^{\circ\prime}$ of phosphate transfer and that for ATP hydrolysis to ADP and P_i:

$$\text{IgG-}P + \text{ADP} \rightleftharpoons \text{IgG} + \text{ATP};\ \Delta G^{\circ\prime} = +0.52 \text{ kcal/mol}$$
$$\text{ATP} + H_2O \rightleftharpoons \text{ADP} + P_i;\ \Delta G^{\circ\prime} = -10.0 \text{ kcal/mol}$$
$$\text{IgG-}P + H_2O \rightleftharpoons \text{IgG} + P_i;\ \Delta G^{\circ\prime} = -9.48 \text{ kcal/mol}$$

At optimal conditions, therefore, the free energy of hydrolysis of Tyr-*P* in IgG-*P* is only slightly lower than that of γ-*P* of ATP. We chose the value of −10 kcal/mol for ATP hydrolysis because in this case the values shown in Table 1 depend so strongly on the quite complex dependence of changes in pH and in Mg^{2+} concentration (16) that the figure −10 kcal/mol appears to be a reasonable approximation. The result for the free energy of hydrolysis of protein-bound Tyr ~ *P* indicates a high phosphate potential (17) similar to that of ATP. In this case we are interested not so much in absolute value as in a comparison with ATP. Even at pH 7.5 and 5 mM Mg^{2+} with K_{eq} of 6.93 a similar calculation of the $\Delta G^{\circ\prime}$ of hydrolysis yields −8.8 kcal/mol—i.e., only −1.3 kcal less than that of ATP, and still quite high.

DISCUSSION

Using the pp60^{src} as kinase, early experiments indicated a rather easy catalysis of reversible *P* transfer between the phosphate of RSV-specific IgG and ADP (Fig. 2). In confirmation, we obtained quite consistent results by using the equilibration of phosphate transfer to TBR-IgG with a fairly wide range of ADP-to-ATP ratios (Table 1); the results turned out to yield, at the pH optimum of 6.5, a release of free energy close to that of the energy-rich γ-*P* in ATP. Thus, as with Ser-*P* (9, 18), the phosphate of Tyr-*P* in a protein turned out to be energy rich.

Some years ago inhibition of enzymatic activity due to formation of a tyrosine phosphate (in this case a phosphodiester) was observed with the adenylylation of bacterial glutamine synthetase (5, 19). In the ornithine decarboxylase of a slime mold, phosphorylation of the enzyme likewise causes a complete inhibition (20). This inhibitory phosphorylation was recently reported to be on tyrosine (21).

The recently reported observation by Huttner (22) that a large number of proteins are sulfurylated, apparently preferentially, on tyrosine is of interest. Tyrosine sulfate incorporation was found also in a pheochromocytoma, and its peak on chromatography was different from that found in CEF (22). Only one protein, fibrinogen, had been found before (23) to contain tyrosine sulfate. Huttner comments on the similarity between sulfurylation and phosphorylation of tyrosine in proteins.

Because the recent observations discussed here call attention to the activity of tyrosine in proteins, observations on tyrosine involvement in binding of DNA to histones in chromatin appear to be of interest. It is reported by Chan and Piette (24) that when the tyrosine is marked with an imidazole spin label, the complexing of DNA in the chromatin remains normal, but complexing is inhibited by denaturation of the histone with urea. Chan and Piette suggest that this effect is due to a disturbance by denaturation of a "burying" of the tyrosine in the normal protein. A similar disturbance of the position of tyrosine might be caused in the enzyme proteins inhibited by phosphorylation.

We thank Dr. R. Feldman, Dr. J. Gregory, Dr. H. Hanafusa, and Dr. J. Sturtevant for their advice and assistance. We are also grateful to Drs. Sturtevant and Epand for permitting us to quote the result of their microcalorimetric determination of ΔH° for Tyr-*P*. This work was supported by Grants PCM7910513 from the National Science Foundation, GM13972-16 from the National Institutes of Health, and BC-340 from the American Cancer Society.

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