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Circular Dichroism Studies on Glycogen Phosphorylase from Rabbit Muscle. Interaction with the Allosteric Activator Adenosine 5′-Monophosphate†

Shoji Shimomura and Toshio Fukui*

ABSTRACT: Circular dichroism (CD) spectra of glycogen phosphorylase from rabbit muscle have been measured in the presence of various ligands, particularly in the near-ultraviolet wavelength region. Phosphorylases *a* and *b* gave similar positive CD spectra as each other, in the 250–310-nm region. The differences in CD between the *a* and *b* forms, as well as the CD changes induced by binding of substrate and other ligands except nucleotides to the enzyme, are all relatively small. Binding of AMP and other nucleotides to phosphorylases *a* and *b*, and $NaBH_4$-reduced phosphorylase *b*, however, induces much larger CD spectral changes than the above. The difference CD curve obtained by subtracting the phosphorylase *b* curve from that of the enzyme–AMP complex is smooth, with a positive maximum at 266 nm and a negative at 289 nm. The results with various other nucleotides show that the induced Cotton effects are dependent on the base chromophore of the nucleotides. The rotational strength of the induced Cotton effect in phosphorylase *b* by AMP increases under various conditions, under which the affinity of the enzyme for AMP is enhanced, e.g., the addition of glucose 1-phosphate, inorganic phosphate, fluoride ion, divalent metal cations, and spermine, low temperatures, and conversion of the enzyme to the *a* form. On the contrary, these factors little affect the induced Cotton effects by IMP, GMP, and dAMP. Amylodextrin gave no effect on the extrinsic Cotton effect by binding of AMP plus Mn^{2+} to phosphorylase *b*, while it did retard the AMP-induced tetramerization of the enzyme. It is suggested that the interaction of nucleotides with phosphorylase involves tacking between the base ring of the bound nucleotides and an a ɔmatic amino acid residue at the allosteric site of the enzyme, ar. ¹ that, in the high affinity form of the enzyme for AMP, particular bondings are newly formed between the enzyme and the nucleotide allowing the heterotropic cooperativity.

Glycogen phosphorylase *b* from rabbit muscle shows an absolute requirement for the allosteric activator AMP[1] for catalytic activity (Cori et al., 1938). The interaction between AMP and phosphorylase *b* has been studied under a variety of conditions by the kinetic, gel filtration, equilibrium dialysis, and calorimetric methods. The affinity of phosphorylase *b* for AMP is enhanced by various ligands, e.g., glucose 1-phosphate, P_i, divalent metal ions, fluoride and sulfate ions, protamine, polylysine, and polyamines (Krebs, 1954; Helmreich and Cori, 1964; Sealock and Graves, 1967; Kastenschmidt et al., 1968; Wang et al., 1968; Mott and Bieber, 1970). It is also affected by a variety of environmental conditions, e.g., kinds of buffer, temperature, and pH (Kastenschmidt et al., 1968), and is enhanced through the enzymatic conversion to the *a* form which is active without AMP (Helmreich et al., 1967). On the other hand, activation of phosphorylase *b* by AMP is known to result from alterations in both V_{max} and K_m for each substrate (see Graves and Wang, 1972).

In addition to AMP, many other nucleotides also activate phosphorylase *b* to different extents (Cori et al., 1938; Okazaki et al., 1968; Black and Wang, 1968, 1970; Mott and Bieber,

† From the Institute of Scientific and Industrial Research, Osaka University, Suita, Osaka 565, Japan. *Received March 30, 1976.*

[1] Abbreviations used: CD, circular dichroism; glucose 1-phosphate or glucose-1-P, α-D-glucopyranose 1-phosphate; P_i, inorganic phosphate; AMP, adenosine 5′-monophosphate; NMR, nuclear magnetic resonance.

1970). However, their effects on the structure and kinetics of phosphorylase *b* differ greatly from the one of AMP in that (1) they do not significantly affect the K_m for glucose 1-phosphate (Black and Wang, 1968), (2) they do not cause the enzyme to associate into tetramer unless the substrate glucose 1-phosphate is present (Appleman, 1962; Black and Wang, 1968, 1970; Ho and Wang, 1973), and (3) they cause different conformational changes of the enzyme from the ones induced by AMP (Mott and Bieber, 1970; Birkett et al., 1971). Further, the affinities of phosphorylase *b* for many AMP analogues are not significantly enhanced by substrates and protamine (Black and Wang, 1968; Mott and Bieber, 1970). These observations indicate that the binding of AMP to the enzyme is strongly affected by the heterotropic interactions, whereas those of the other nucleotides are little affected.

Although considerable information concerning the structure of a number of proteins in solution can be obtained from measurements of their optical activity, the technique has not been sufficiently used to study conformation and interaction of phosphorylase (Hedrick, 1966; Johnson and Graves, 1966; Kamogawa et al., 1968; Johnson et al., 1970). This paper presents the results of the CD studies on the interaction of phosphorylase with AMP and other ligands, particularly in the near-ultraviolet wavelength region. The data suggest that the interaction of nucleotides with phosphorylase involves stacking between the base ring of the bound nucleotides and an aromatic amino acid residue at the allosteric site of the enzyme, and that, in addition to the above interaction, particular bondings are newly formed between AMP and phosphorylase when the enzyme in its low affinity form for the nucleotide is transformed to the high affinity form.

Materials and Methods

The three times crystallized phosphorylase *b* was isolated from rabbit muscle by the procedure of Fischer and Krebs (1958). Phosphorylase *a* was prepared from crystalline phosphorylase *b* and phosphorylase kinase by Mr. M. Ariki of our laboratory, according to Krebs and Fischer (1962). $NaBH_4$-reduced phosphorylase *b* was prepared by the method of Johnson et al. (1970). Residual AMP and other compounds contaminated were removed by treating the enzyme with charcoal and Sephadex G-25. AMP and dAMP were obtained from Sigma Chemical Co. IMP, GMP, CMP, ADP, cAMP, and adenosine were purchased from Kohjin Co. Spermine tetrahydrochloride, shellfish glycogen, glucose 1-phosphate, and glucose were obtained from Nakarai Chemicals. Amylodextrin ($\overline{DP}$, 10–15) was a gift from Mr. K. Kato of this Institute.

CD measurements were carried out with a Jasco spectropolarimeter, Model J-20, equipped with a CD attachment, which was calibrated with *d*-camphor-10-sulfonic acid (Cassim and Yang, 1969; DeTar, 1969). Molecular ellipticity, $[\theta]_M$, and mean residue ellipticity, $[\theta]_{mrw}$, were obtained from the following equations, $[\theta]_M = 100\theta/LM$ and $[\theta]_{mrw} = 100\theta/Lm$, in units of deg cm^2 dmol^{-1}, respectively, where θ is the ellipticity in degrees as obtained from the difference between in the presence and absence of protein, L is the light path length in cm, and M and m are the molar concentration of protein and the mean residue molar concentration, respectively. A molecular weight of 100 000 for phosphorylase monomer (Cohen et al., 1971) or a mean residue weight of 116 was used in the calculation of the ellipticity. A 1-mm path length cell was usually used in the far-ultraviolet wavelength region at 0.1 mg/ml protein, and in the near-ultraviolet region at 10 mg/ml protein. A 10-mm cell was used in the near-ultraviolet region at 1–2 mg/ml protein. In some experiments, 5- and 20-mm cells were also used. The temperature was controlled by means of a thermostated cell holder, to which water at a constant temperature was circulated.

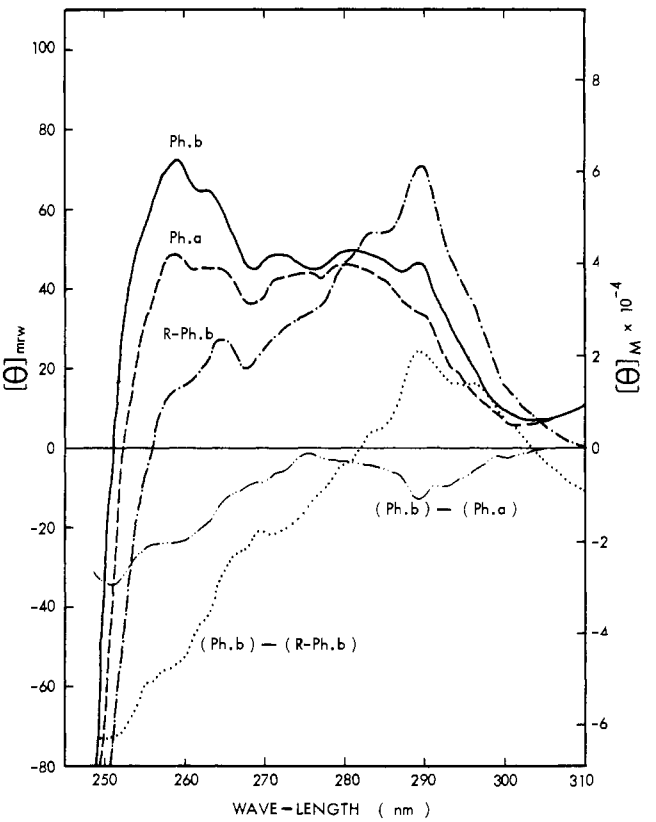

FIGURE 1: The CD spectra of rabbit muscle phosphorylase in the near-ultraviolet region in 50 mM Tris-HCl–0.1 M KCl–1 mM 2-mercaptoethanol, pH 7.5. (—) Phosphorylase b (11 mg/ml) at 25 °C; (- - -) 0.85 mg/ml phosphorylase *a* at 25 °C; (— · —) 11 mg/ml $NaBH_4$-reduced phosphorylase *b* in the presence of 6 mM spermine at 20 °C; (— · · —) the difference CD spectrum of enzyme *a* − enzyme *b*; (. . .) the difference CD spectrum of reduced enzyme *b* − enzyme *b*.

Protein concentration was determined spectrophotometrically using an absorbance index of 13.2 for a 10 mg/ml solution at 280 nm (Kastenschmidt et al., 1968). Nucleotide concentration was also determined spectrophotometrically. Ultracentrifugal runs were performed on a Hitachi 282 analytical ultracentrifuge at a rotor speed of 60 000 rpm and a temperature of 27 °C. Sedimentation coefficients were corrected for viscosity and density of the buffer, regarded as 0.2 M KCl, to water at 20 °C.

Results

Circular Dichroism of Phosphorylase. The CD spectra of phosphorylases *a* and *b* and $NaBH_4$-reduced phosphorylase *b* in the near-ultraviolet wavelength region are shown in Figure 1. The ellipticity values are normalized to the mean residue weight of protein and also per decimole of enzyme monomer and represented as $[\theta]_{mrw}$ and $[\theta]_M$, respectively. The CD spectrum of phosphorylase *b* at neutral pH has at least five positive dichroic bands in the 250–310-nm region, namely at 259, 263, 272, 281, and 289 nm. The differences in measurements at different enzyme concentrations from 1 to 10 mg/ml are within a limit of experimental errors. The peaks at 259, 281, and 289 nm may be in error to ±2, ±4, and ±2 deg cm^2 dmol^{-1}. The CD spectra of phosphorylases *a* and *b* have strong resemblances in spite of the fundamental difference in their function, whereas $NaBH_4$-reduced phosphorylase *b* shows a

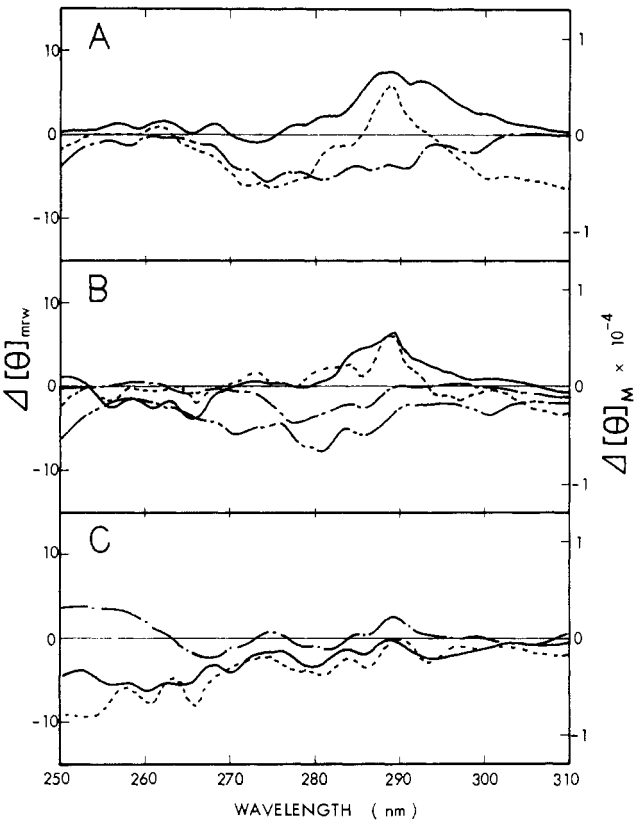

FIGURE 2: The difference CD spectra of phosphorylases *a* and *b* obtained by subtracting the enzyme curve from that in the presence of various ligands in 50 mM Tris-HCl–0.1 M KCl–1 mM 2-mercaptoethanol, pH 7.5 at 25 °C. (A) Phosphorylase *a*, 0.85 mg/ml: (—) 1% amylodextrin; (- - -) 90 mM glucose; (— · —) 0.1 M P_i. (B) Phosphorylase *b*, 0.81 mg/ml: (—) 1% amylodextrin; (- - -) 50 mM glucose; (— · —) 0.1 M P_i; (— · · —) 50 mM glucose 1-phosphate. (C) Phosphorylase *b*, 0.81 mg/ml: (— · —) 0.5 M KF; (—) 20 mM $MnCl_2$; (- - -) 10 mM spermine.

relatively different spectrum from that of the intact enzyme.

In the 300–400-nm region is shown a smooth positive CD spectrum of phosphorylase *b* centered at 335 nm, which originates from the bound pyridoxal 5′-phosphate chromophore absorbing at 333 nm (Johnson and Graves, 1966). The molar ellipticity of this dichroic band (2.6×10^4 deg cm^2 dmol^{-1}) is of the same order as those appearing in the 250–310-nm region. Phosphorylase *a* shows nearly the same absorption and dichroic bands around 335 nm, whereas $NaBH_4$-reduced phosphorylase *b* at pH 7.5 has no prominent bands in both absorption and CD above 310 nm. By studying the spectra of $NaBH_4$-reduced phosphorylase *b*, Johnson et al. (1970) have concluded that the covalently bound pyridoxamine 5′-phosphate on the reduced enzyme above pH 7 has the neutral tautomer form in which the oxygen in position 3 of the pyridine ring is protonated and the pyridine nitrogen is unprotonated, and which would be expected to absorb at 290 nm. The observed increment of the 290-nm ellipticity band upon the reduction of phosphorylase *b* (see Figure 1) would be explained by the appearance of a new optically active band corresponding to the tautomer form.

Effects of Ligands on the Circular Dichroism of Phosphorylase. The function of phosphorylase is influenced by a number of ligands including substrates. The effects of these ligands on the CD spectra of phosphorylases *a* and *b* in the 250–310-nm region are shown in Figure 2. Based on the kinetic constants, the enzymes are nearly saturated with the ligands under these conditions. The difference spectra generated when amylodextrin (substrate) and glucose (a competitive inhibitor with respect to glucose 1-phosphate; Cori et al., 1943) are bound to phosphorylases *a* and *b* show increments near 290 nm. On the other hand, the spectral changes upon binding of glucose 1-phosphate and P_i show decrements in the 260–300-nm region. It is important to note that quite similar CD changes are observed among phosphorylases *a* and *b*. These results indicate that the conformational perturbations induced by substrate binding to the active and inactive forms of phosphorylase are similar, and further suggest that the extent of the rearrangement of the substrate binding site in accordance with activation of the inactive enzyme is not spacious.

Phosphorylase *b* exists as dimer regardless of the presence of substrate (see Graves and Wang, 1972). On the other hand, amylodextrin and glucose have been shown by Wang et al. (1965) and Metzger et al. (1967) to cause dissociation of phosphorylase *a* tetramer to dimer. Therefore, the difference spectrum shown in Figure 2A may include the difference between phosphorylase *a* tetramer and its dimer. Since there are good similarities in the spectra of phosphorylases *a* and *b* in the presence of amylodextrin and glucose (Figure 2A, B), it is concluded that the CD perturbation induced by tetramerization is also very minor. Figure 2C shows the effects, on the CD spectra of phosphorylase *b*, of Mn^{2+}, F^-, and spermine, all of which enhance the affinity of phosphorylase *b* for AMP. Thus, the ellipticity change induced by binding of any ligand applied is of the same order as the difference ellipticity between phosphorylases *a* and *b*.

The CD spectra of phosphorylases *a* and *b*, and of $NaBH_4$-reduced phosphorylase *b* in the presence of AMP in the 250–310-nm region show marked changes due to the activator. Free AMP has an absorption band, as well as a small negative ellipticity band (-4×10^3 deg cm^2 dmol^{-1}), near 260 nm (Brahms et al., 1966). This ellipticity is much less than that of phosphorylase in this wavelength region. The difference curve obtained by subtracting the phosphorylase *b* curve from that of the phosphorylase *b*–AMP complex is smooth, with a positive maximum at 266 nm and a negative at 289 nm (Figure 3A). Figure 3C shows the ellipticity changes which occurred upon binding of the AMP-analogues possessing the unmodified adenine ring, e.g., dAMP, ADP, adenosine, and cAMP. The difference CD curve obtained by binding of dAMP is fairly similar to that of AMP; it has two transitions of opposite sign in a wavelength region similar to that of AMP. Contrarily, the AMP analogues with other kinds of base than adenine, e.g., GMP and IMP, induce different types of dichroism changes, as shown in Figure 3B. However, dichroic changes induced from complexing with ADP, cAMP, adenosine, and CMP are comparable to the noise level under the conditions used, and are not explored in more detail. Experiments using higher concentrations of these ligands are difficult because of the large absorptions in these wavelength regions. The difference CD curves obtained with $NaBH_4$-reduced phosphorylase *b* by complexing with AMP, dAMP, GMP, and IMP (Figure 3E) resemble the corresponding curves in the intact enzyme. Two similar dichroic bands to those of phosphorylase *b*–AMP were obtained with phosphorylase *a* upon binding of the nucleotide (Figure 3D).

The positions of the induced Cotton effects are close to the wavelength of the absorption band of each free nucleotide; e.g., free AMP and dAMP absorbing at 259 nm show the induced Cotton effect by binding of the nucleotides around 265 nm, free IMP absorbing at 250 nm gives the induced Cotton effect around 250–260 nm, and free GMP having a peak at 253 nm and a shoulder around 275 nm shows the induced Cotton effect

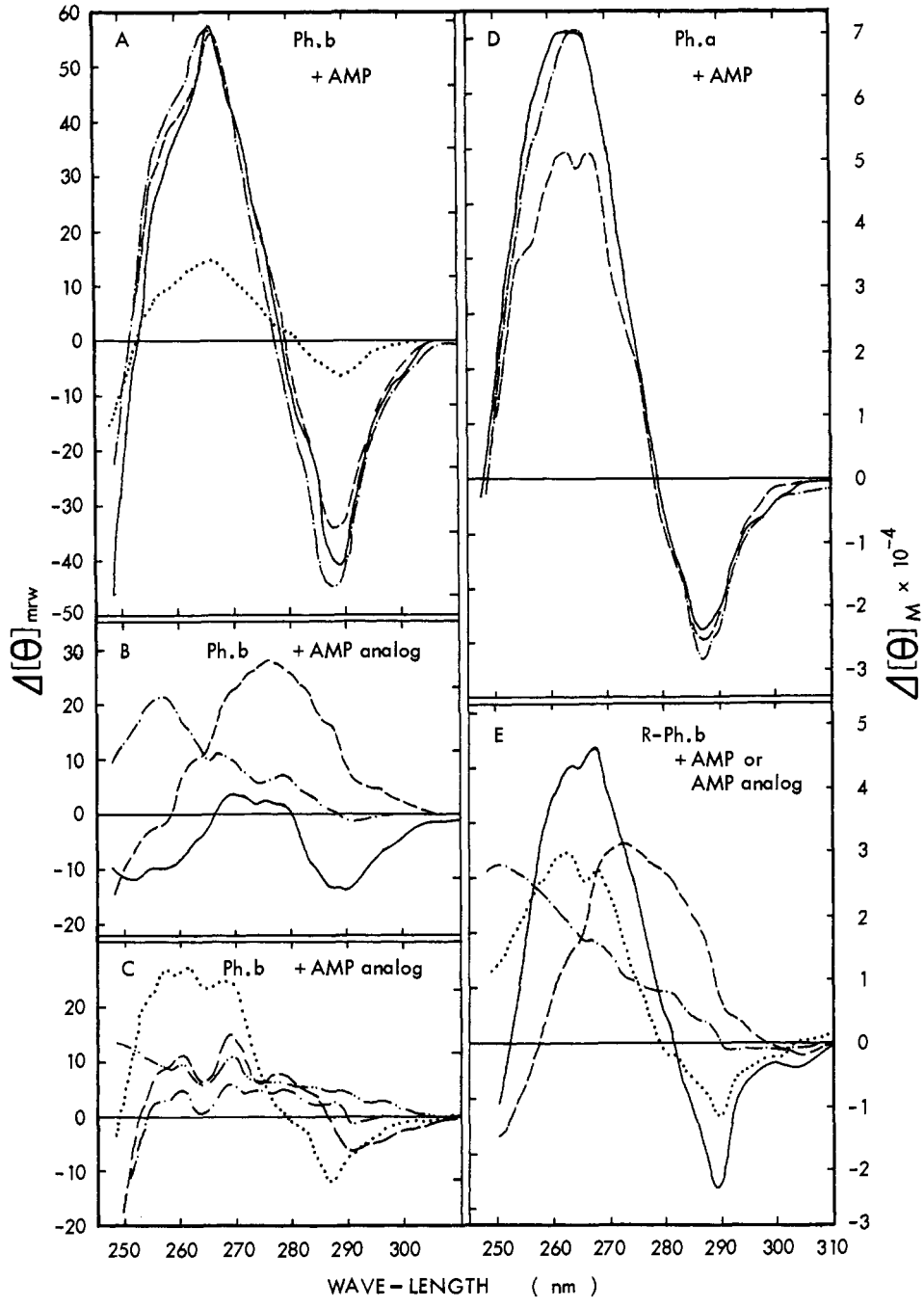

FIGURE 3: The difference CD spectra of phosphorylases obtained by subtracting the enzyme curve from that in the presence of nucleotides in 50 mM Tris-HCl–0.1 M KCl–1 mM 2-mercaptoethanol, pH 7.5 at 25 °C. (A) Phosphorylase *b*: (···) (11 mg/ml enzyme plus 0.2 mM AMP) − (11 mg/ml enzyme); (—) (11 mg/ml enzyme plus 0.2 mM AMP plus 20 mM $MnCl_2$) − (11 mg/ml enzyme); (- - -) (10 mg/ml enzyme plus 0.18 mM AMP plus 5.5 mM spermine) − (10 mg/ml enzyme plus 5.5 mM spermine); (— · —) (10 mg.ml enzyme plus 1 mM AMP plus 20 mM $MnCl_2$ plus 1% amylodextrin) − (10 mg/ml enzyme plus 1% amylodextrin). (B) Phosphorylase *b*, 10 mg/ml, in the presence of 5.5 mM spermine and: (—) 1 mM CMP; (- - -) 0.9 mM GMP; (— · —) 1.7 mM IMP. (C) The same as in B: (- - -) 1.5 mM ADP; (— · —) 1.5 mM adenosine; (— ·· —) 1.5 mM cAMP; (···) 0.85 mM dAMP. (D) Phosphorylase *a*, 0.85 mg/ml: (—) (enzyme plus 0.1 mM AMP) − (enzyme); (- - -) (enzyme plus 0.1 mM AMP plus 0.1 M P_i) − (enzyme plus 0.1 M P_i); (— · —) (enzyme plus 0.1 mM AMP plus 1% amylodextrin) − (enzyme plus 1% amylodextrin). (E) $NaBH_4$-reduced phosphorylase *b*, 11 mg/ml, in the presence of 6 mM spermine and: (—) 1 mM AMP; (- - -) 0.99 mM GMP; (— · —) 1.9 mM IMP; (···) 0.94 mM dAMP.

around 275 nm. The dichroic bands of phosphorylase itself in the 250–310-nm region may originate from the electron transitions of the aromatic groups (tryptophan, tyrosine, and phenylalanine) and possibly of the bound pyridoxal 5′-phosphate. Therefore, the ellipticity change represented by binding of any ligand having no absorption band in this region may arise only from the perturbation of the asymmetric environment of the protein chromophores. The contribution of the ellipticity change by the perturbation of the protein chromophores generated by binding of nucleotides to the enzyme could be expected to be in a similar magnitude to the ellipticity changes by binding of substrates as well as the difference ellipticity between phosphorylases *a* and *b*. The observed ellipticity changes by binding of the nucleotides, however, are much larger than those expected for protein perturbation, and have a dependence on the kinds of base chromophore.

Effect of the Additional Ligands on the Induced Dichroic Bands from Phosphorylase–AMP Complex. The concentra-

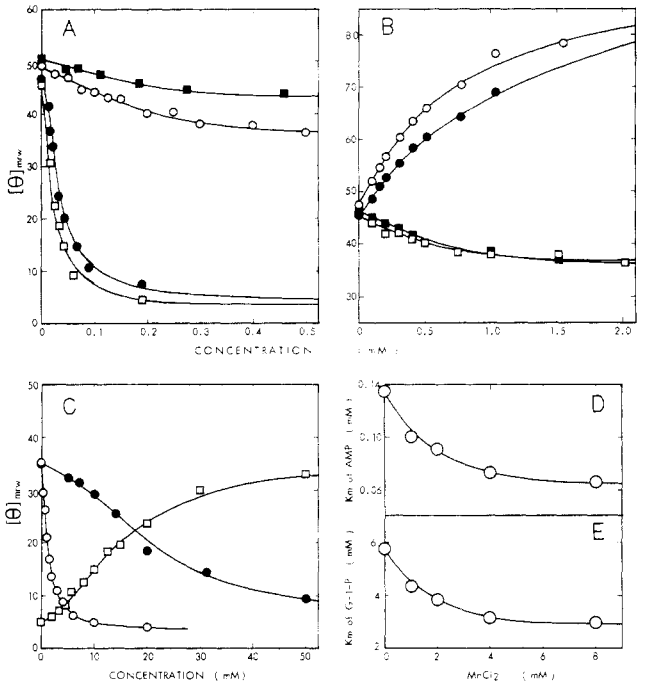

FIGURE 4: The CD titrations by the addition of ligands in 50 mM Tris-HCl–0.1 M KCl–1 mM 2-mercaptoethanol, pH 7.5 at 25 °C. Phosphorylase *b*, 0.9–1.2 mg/ml for AMP and dAMP titrations, and 8.2 mg/ml for GMP titration. (A) AMP titration at 289 nm: (○) in the absence of additional ligands; (●) in the presence of 0.5 M KF; (□) of 20 mM $MnCl_2$; (■) of 50 mM glucose. (B) GMP and dAMP titrations at 275 and 288 nm, respectively: (○) GMP; (●) GMP in the presence of 10 mM spermine; (□) dAMP; (■) dAMP in the presence of 10 mM spermine. (C) $MnCl_2$, P_i, and glucose titrations in the presence of AMP at 289 nm; (○) $MnCl_2$ in the presence of 1 mM AMP; (●) P_i in the presence of 1 mM AMP; (□) glucose in the presence of 0.5 mM AMP plus 20 mM $MnCl_2$. (D) The K_m values for AMP in the presence of $MnCl_2$. The assays were performed in the direction of glycogen synthesis in 50 mM sodium maleate–50 mM 2-mercaptoethanol–1% glycogen–20 mM glucose-1-phosphate phosphorylase *b* at pH 6.8 and 30 °C. (E) The K_m values for glucose 1-phosphate in the presence of $MnCl_2$. The assays were performed as in D, except that the AMP concentration was fixed at 1 mM and the glucose 1-phosphate concentration was varied.

tion dependence of the effect of the nucleotides on phosphorylase *b* is examined to study further the characteristics of the induced Cotton effect observed. Experimental results are shown in Figure 4A–C. The apparent dissociation constants were calculated from these results using the following Hill-type equation

$$\log \frac{[\theta]_O - [\theta]_C}{[\theta]_C - [\theta]_\infty} = n(\log C - \log K_d)$$

where $[\theta]_O$, $[\theta]_C$, and $[\theta]_\infty$ are the mean residue ellipticity at the fixed wavelength in the absence of ligand, the ones in the presence of ligand at a concentration of C, and the extrapolated value of the ellipticity to the infinite concentration of ligand, respectively, and n, C, and K_d are the Hill coefficient, the concentration of free ligand, and the apparent dissociation constant of phosphorylase *b* and ligand, respectively. The concentration of free ligand was calculated using the following equation

$$C = C_t - \frac{[\theta]_O - [\theta]_C}{[\theta]_O - [\theta]_\infty} E_t$$

where C_t and E_t are total concentrations of ligand and enzyme monomer, respectively.[2] Wavelengths of 289, 288, and 275 nm were chosen for measurement of ellipticities in the cases of AMP, dAMP, and GMP, respectively, since, at these wavelengths, the ellipticities of free nucleotides are sufficiently small (molar ellipticities of an order of -10^2 deg cm^2 dmol^{-1}), while the maximum ellipticity changes are obtained on the complex formation. Experiments with other nucleotides are difficult because of their low affinity to the enzyme and large absorptions at high concentrations. The values of the apparent dissociation constants, the maximum ellipticity changes $\Delta[\theta]$ (= $[\theta]_\infty - [\theta]_O$), and the Hill coefficients obtained from the data in Figure 4 are listed in Table I.

TABLE I: Apparent Dissociation Constants of Rabbit Muscle Phosphorylase *b*–Nucleotide or Other Ligand Complexes Calculated from CD Titration.[a]

Ligand	Additional ligands	K_d (mM)	$\Delta[\theta]$	n
AMP		0.17	−15	1.3
AMP	20 mM $MnCl_2$	0.022	−42	1.8
AMP	0.5 M KF	0.034	−43	1.7
AMP	50 mM glucose	~0.3	~−10	
AMP	10 mM spermine + 50 mM glucose	0.13	−16	1.4
GMP		0.60	+42	1.1
GMP	20 mM glucose-1-P	0.76	+36	1.0
GMP	10 mM spermine	0.83	+42	1.1
GMP	0.5 M KF	>1		
GMP	50 mM glucose	0.56	+49	1.2
dAMP		0.45	−11	1.3
dAMP	10 mM spermine	0.62	−13	1.2
$MnCl_2$	1 mM AMP	1.2	−32	1.5
P_i	1 mM AMP	23	−32	1.8
Glucose	0.5 mM AMP + 20 mM $MnCl_2$	20	+37	1.5

[a] The dissociation constant (K_d), the maximum ellipticity change ($\Delta[\theta] = [\theta]_\infty - [\theta]_0$), and the Hill coefficient (n) were calculated from the data of Figure 4 by using the Hill-type equation (see Results).

The apparent dissociation constant thus obtained for AMP in the absence of other ligands is 0.17 mM, which is in good agreement with the values obtained in other laboratories by the techniques of gel filtration, equilibrium dialysis, calorimetric study, and so on (Kastenschmidt et al., 1968; Avramovic and Madsen, 1968; Mott and Bieber, 1970; Wang et al., 1970). In the presence of Mn^{2+} and F^-, the observed dissociation constants decrease to 22 and 34 μM, respectively. These results agree well with the results of previous investigations, which showed enhanced affinity of phosphorylase *b* for AMP by divalent cations and F^- (Kastenschmidt et al., 1968; Sealock and Graves, 1967). Glucose decreases the binding of AMP to phosphorylase *b*. The CD titration curve in the presence of 50 mM glucose is rather biphasic, in which increasing the concentration of AMP up to 1 mM results in a decrease of the ellipticity at 289 nm, but the concentration over 1 mM causes a slight increase. This biphasic nature of the curve may have arisen from binding of AMP to the second low affinity site on phosphorylase *b* as shown by Wang et al. (1970). Spermine has been shown by Wang et al. (1968) to enhance the affinity of phosphorylase *b* for AMP. It has an effect of enhancing the AMP affinity even in the presence of glucose (Table I). It is thus concluded that the observed extrinsic Cotton effects with AMP are due to binding of the nucleotide at the proper allosteric site of phosphorylase *b*.

The maximum ellipticity change ($\Delta[\theta]$) at 289 nm upon binding of AMP to phosphorylase *b* increases from −15 to −42 and −43 in accordance with the addition of Mn^{2+} and F^- respectively, and it decreases to −10 in the presence of glucose

[2] This relationship is only valid if the change in CD spectrum is proportional to the saturation curve.

(Table I). Glucose 1-phosphate, Mg^{2+}, Co^{2+}, and P_i give a similar increasing effect to the case of Mn^{2+} and F^-, and the addition of amylodextrin does not affect the ellipticity at 289 nm, though the data are not shown in the table. On the other hand, the shapes of the whole difference CD spectra are not significantly changed in the presence of additional ligands (Figure 3A). Thus, only the rotational strength of the induced Cotton effect from binding with AMP is affected by the formation of the ternary complexes. Figure 4C shows the dependence of the ellipticities of the enzyme–AMP complex at 289 nm upon concentrations of the additional ligands. The apparent dissociation constants of those ligands were calculated from these data in a similar manner to the case of AMP, and are listed in Table I. The apparent dissociation constant calculated for P_i, 23 mM, agrees well with the reported K_m value for P_i, 15 mM (Engers et al., 1969, 1970).

$MnCl_2$ enhances the activity of phosphorylase *b* at a low concentration of AMP or glucose 1-phosphate. The apparent K_m values for AMP and glucose 1-phosphate decrease with an increase of $MnCl_2$ concentration, as shown in Figure 4D, E, whereas the Hill coefficients and V_{max} values are independent of the salt concentration. Glycogen kinetics are also not affected by $MnCl_2$ concentration. An apparent dissociation constant of the salt was calculated from the relation between the K_m values for AMP and for glucose 1-phosphate and $MnCl_2$ concentration, as 1.5 mM. This value is comparable to the one calculated from the CD titration, 1.2 mM. Other divalent cations also decrease the K_m values for AMP and for glucose 1-phosphate; the apparent dissociation constants were calculated as 3.2, 2.7, 2.9, and 2.4 mM for $MgCl_2$, $CaCl_2$, $SrCl_2$, and $BaCl_2$, respectively. Birkett et al. (1971) showed in the study of proton relaxation enhancement that the dissociation constants of Mn^{2+} bound close to the AMP binding site (Bennick et al., 1971) are 0.17 mM at pH 8.5 and about 0.4 mM at pH 7.5, and that the bound Mn^{2+} gives no effect on the AMP affinity of the enzyme. Thus, it appears that the bound Mn^{2+} detected in their study is different from the one studied through the CD and kinetics.

The apparent dissociation constants calculated from the CD titrations of GMP and dAMP, which are less effective activators than AMP, are largely in good agreement with the previous values (Black and Wang, 1970; Mott and Bieber, 1970; Okazaki et al., 1968). However, the substances increasing the affinity for AMP, e.g., glucose-1-phosphate, spermine, and KF, cause only a slight effect on the affinity for GMP and dAMP, in contrast to the case of AMP, with a tendency of resulting in even larger values of the dissociation constants (Table I). Glucose which decreases the affinity for AMP causes a little increase in the affinity for GMP. On the other hand, there is little difference in the maximum ellipticity changes of the enzyme–GMP or –dAMP complex with or without additional ligands, as compared with large differences in the case of the enzyme–AMP complex.

Influence of Temperature on the Induced Dichroic Bands from Phosphorylase b–AMP Complex. The temperature dependence of the induced dichroic bands from phosphorylase *b*–AMP complex was studied since the affinity of the enzyme for AMP has been known to be significantly affected by temperature (Kastenschmidt et al., 1968; Ho and Wang, 1973). The enzyme alone has no observable variance in the ellipticities at 266 and 289 nm at different temperatures. On the other hand, the enzyme in the presence of 1 mM AMP shows a marked temperature dependence. The ellipticity changes at both wavelengths increase with a decrease in temperature, giving a sigmoidal curve with an inflexion point around 13 °C:

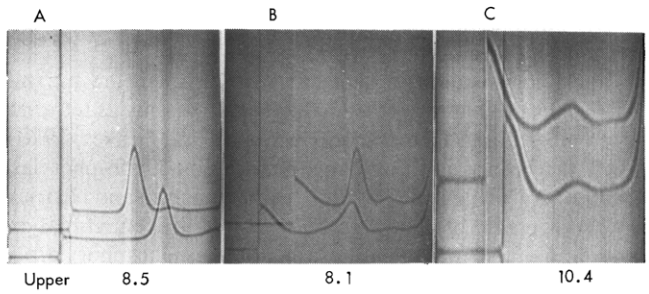

FIGURE 5: The sedimentation patterns of phosphorylase *b* in the presence of AMP and other additional ligands. (A) Enzyme (9 mg/ml) in the absence (upper curve) and presence (lower curve) of 1 mM AMP plus 20 mM $MnCl_2$. (B) Enzyme (9 mg/ml) in the presence of 1% amylodextrin (upper curve) and of 1% amylodextrin plus 1 mM AMP plus 20 mM $MnCl_2$ (lower curve). (C) Enzyme, 3.8 mg/ml (upper curve) and 1.9 mg/ml (lower curve), in the presence of 1% amylodextrin plus 1 mM AMP plus 20 mM $MnCl_2$. The numerical figures under the Schlieren patterns indicate the sedimentation coefficient values ($s_{20,w}$ in S of each main peak).

$\Delta[\theta]_{mrw}$ at 266 nm = 22, 36, and 48; $\Delta[\theta]_{mrw}$ at 289 nm = −14, −21, and −30, at 30, 15, and 5.8 °C, respectively. This increment may not reflect the increase in the concentration of the enzyme–AMP complex since the enzyme is almost saturated by AMP under these conditions (K_d at 25 °C = 0.17 mM; see Table I). Formation of the ternary complex with the second ligand, e.g., spermine and Mn^{2+}, induced a large ellipticity change even at high temperatures, and the change is increased only slightly with a decrease in temperature: in the case of 1 mM AMP plus 6 mM spermine, $\Delta[\theta]_{mrw}$ at 266 nm = 52, 54, and 57; $\Delta[\theta]_{mrw}$ at 289 nm = −35, −37, and −39, at 25, 15, and 5.8 °C, respectively. Thus, it is apparent that low temperatures have a similar effect, on the addition of the second ligands, of increasing the induced ellipticity change.

Relation between the Induced Dichroic Bands and Tetramerization of Phosphorylase. As described in the preceding sections, the rotational strength of the extrinsic Cotton effect induced by binding of AMP to phosphorylase *b* is increased by various factors which enhance the affinity of the enzyme for AMP, and is decreased by the addition of glucose. It is noted, however, that association of phosphorylase *b* dimer to tetramer will also occur under those conditions, e.g., the enzyme plus both AMP and the divalent cations, fluoride ion, spermine, glucose 1-phosphate, or P_i, and the enzyme plus AMP at low temperatures (Helmreich and Cori, 1964; Black and Wang, 1968; Wang et al., 1968, 1970; Assaf and Yunis, 1970). To clarify the relation between the induced dichroic bands and the tetramerization of the enzyme, sedimentation velocity and CD were measured under the same conditions. The results are shown in Figure 5. Free phosphorylase *b* at 27 °C exists mainly as the dimer species which sediments at 8.5 S (Figure 5A, the upper curve). In the presence of 1 mM AMP plus 20 mM $MnCl_2$, the enzyme associates almost completely to the tetramer species with 13.2 S (Figure 5A, the lower curve). On the subsequent addition of amylodextrin to this system, a significant decrease of the sedimentation constant and a considerable broadening of the peak occurred (Figure 5B, the lower curve). Lowering the enzyme concentration in a similar mixture resulted in a further decrease in the *s* value of the main peak (Figure 5C). These results indicate that amylodextrin retards the AMP-induced association of phosphorylase *b*, the effect being more prominent at lower enzyme concentrations. On the other hand, the polysaccharide gave no change in the extrinsic Cotton effect induced by binding of AMP and Mn^{2+} to the enzyme (Figure 3A) and the ellipticity

at 289 nm does not show any dependence on the enzyme concentration (data not shown).

Phosphorylase *a* at a concentration of about 0.8 mg/ml, which was used in the present study, exists mainly as tetramer (DeVincenzi and Hedrick, 1970; Huang and Graves, 1970). AMP has been shown to enhance dissociation of phosphorylase *a* tetramer (Wang and Graves, 1964; DeVincenzi and Hedrick, 1970). Metzger et al. (1967) showed by ultracentrifugal examination that amyloheptaose dissociates phosphorylase *a* tetramer to dimer. Amylodextrin, however, has no effect on the induced Cotton effect of phosphorylase *a* as in the case of phosphorylase *b*, as shown in Figure 5D. It is thus concluded that the observed enhancement in the extrinsic Cotton effect is caused by different states of the AMP binding at the allosteric site, not by association of the enzyme to tetramer.

Effects of AMP on the Circular Dichroism Spectra of Pyridoxal 5′-Phosphate. The CD spectrum above 300 nm originated from bound pyridoxal 5′-phosphate in phosphorylase *b* is slightly altered by binding of AMP. The molar ellipticity changes at 335 nm on the addition of 1 mM AMP, of 1 mM AMP plus 6 mM spermine, and of 1 mM AMP plus 6 mM spermine plus 1% amylodextrin to phosphorylase *b* at a concentration of 2.4 mg/ml are +300, +3700, and +1100 deg cm^2 $dmol^{-1}$, respectively. When the temperature is decreased from 25 to 15 and to 5.8 °C, the molar ellipticity changes on the addition of 1 mM AMP alone increase from +300 to +600 and +1900 deg cm^2 $dmol^{-1}$, respectively. These increments may be related to the tetramerization of phosphorylase *b*. The CD spectra in the 200–250-nm wavelength region of phosphorylase *b* in the presence of 0.2 mM AMP plus 6 mM spermine are almost the same as that in their absence.

Discussion

Binding of AMP and Other Nucleotides. Glycogen phosphorylase from rabbit muscle contains 14 tryptophan, 37 tyrosine, 38 phenylalanine residues, and a pyridoxal 5′-phosphate group, but no cystine residue, per monomer of molecular weight 100 000 (Cohen et al., 1973). Therefore, the dichroic bands observed in the 250–300-nm region may be originated from the transitions of the aromatic amino acid residues and possibly of the pyridoxal 5′-phosphate. The ellipticity changes in this wavelength region by binding of ligands having no chromophore and by the dissociation of the enzyme tetramer to dimer were shown to be very slight, less than a mean residue ellipticity of 10 deg cm^2 $dmol^{-1}$, and to be in the same extent as in the difference ellipticity between phosphorylases *a* and *b*. On the other hand, the ellipticity changes induced from binding of nucleotides were much larger than those; the maximum mean residue ellipticity changes induced from binding of AMP, GMP, and dAMP to phosphorylase *b* are approximately 60, 50, and 40 deg cm^2 $dmol^{-1}$, respectively. They also have a strong dependence on the kinds of base chromophores. The positions of the induced Cotton effects were also very close to the wavelength of the absorption band of each free nucleotide. Therefore, these observations could not be interpreted merely by the CD perturbation induced by the conformational changes of the microenvironments of the protein chromophores.

Purine nucleotides and nucleosides in solution have small negative ellipticity bands, the molar ellipticities being on an order of 10^3 deg cm^2 $dmol^{-1}$, in the near-ultraviolet wavelength region (Miles et al., 1969; Warshaw and Cantor, 1970). The near-ultraviolet Cotton effects are usually centered in the region of their respective absorption maximum. NMR studies show that purine nucleotides favor the anti conformation in solution (Schweizer et al., 1968; Danyluk and Hruska, 1968).[3] However, as purine nucleosides have a greater freedom of rotation around the glycosidic linkage compared with pyrimidine nucleosides, the observed magnitudes of the Cotton effects of purine nucleosides are much smaller than those of pyrimidine nucleosides (Emerson et al., 1966, 1967; Yang et al., 1966). Indeed, the magnitudes of the Cotton effects of cyclic adenosine analogues which have a fixed anti or syn conformation are much larger, the molar ellipticities being on an order of 10^4 deg cm^2 $dmol^{-1}$, than those of noncyclic derivatives (Miles et al., 1967; Ikehara et al., 1971). If protein could bind preferentially a specific form among optical isomers, anti or syn, the form is concentrated over the counterpart, and, as a result, its CD spectrum would appear in the CD difference spectrum between the enzyme plus the nucleotide and the enzyme alone. This interpretation has been made on several systems in which proteins interact with nucleotides (Oshima and Imahori, 1971; Girault et al., 1973; Leary et al., 1975). If such an interpretation is applied to the phosphorylase–nucleotide system, it is possible that the IMP is fixed to the syn conformation in the allosteric site since the extrinsic Cotton effects induced by binding of the nucleotide to native and $NaBH_4$-reduced phosphorylase *b* having one positive ellipticity band around 250 nm are quite similar to the Cotton effect of the syn conformation of the nucleotide.[3] The extrinsic Cotton effects induced from binding of AMP, dAMP, and GMP to phosphorylase, however, differ significantly from the Cotton effects of either syn or anti conformation of the nucleotides (Miles et al., 1967; Guschlbauer and Courtois, 1968; Oshima and Imahori, 1971; Ikehara et al., 1971).

Another interpretation proposed on the extrinsic Cotton effects induced from binding of nucleotides to phosphorylase which we favor at present is that those Cotton effects observed arise from the exciton splitting and the interactions between near- and far-ultraviolet transitions by stacking of the base chromophore of the nucleotide and an aromatic chromophore of the protein. This interpretation has been deduced for successful application to the stacking of dinucleotides (Tinoco, 1964; Bush and Brahms, 1967) and has been applied also to the extrinsic Cotton effects induced from binding of nucleotides on ribonuclease (Sander and Ts'o, 1971; Yoshida et al., 1971). On the other hand, Anderson and Graves (1973) and Anderson et al. (1973) have shown that the AMP binding site in phosphorylase *b* is a rather large hydrophobic region since the adenine derivatives substituted at the C-8 by highly hydrophobic groups are effective competitive inhibitors with respect to AMP. From x-ray studies, several other proteins have been shown to have the aromatic groups very close to the base of the nucleotides when they complex with nucleotides: RNase-S (Richards et al., 1970), staphylococcal nuclease (Cotton and Hazen, 1971), glyceraldehyde-3-phosphate dehydrogenase (Buehner et al., 1974), lactate dehydrogenase (Adams et al., 1973), and flavodoxin (Watenpaugh et al., 1972).

These results support the proposed idea of stacking between the base of the bound nucleotide and an aromatic group of phosphorylase. A possibility of the involvement of bound pyridoxal 5′-phosphate would be excluded since the extrinsic Cotton effects by binding of AMP to native and $NaBH_4$-reduced phosphorylase *b* are very similar. As the electronic

[3] Since the very recent report by Chachaty et al. (Chachaty, C., Zemb, T., Langlet, G., Tran Dinh Son, Buc, H., and Morange, M. (1976), *Eur. J. Biochem. 62*, 45) showed that the conformations of free nucleotides in solution are controversial, the relationship between the CD spectra and the conformations should be reexamined.

transition of the bound pyridoxamine chromophore is completely different from that of the bound pyridoxal chromophore of phosphorylase *b*, the extrinsic Cotton effects on both enzymes should be distinguishable from each other, if the pyridoxal 5′-phosphate is stacked with the base of the nucleotide. Further, apophosphorylase *b* which has been resolved from pyridoxal 5′-phosphate still gave a similar extrinsic Cotton effect to that of the holoenzyme, upon binding of AMP (unpublished results). Therefore, it is very likely that the aromatic group which is stacked with the base of nucleotides is of the aromatic amino acid residue, i.e., tryptophan, tyrosine, or phenylalanine, in phosphorylase.

Heterotropic Interaction. The magnitude of the extrinsic Cotton effects induced from binding of AMP to phosphorylase was highly dependent on the conditions, although the spectrum was not significantly altered in its shape. The rotational strength of the Cotton effect was enhanced under the conditions in which the affinity of the enzyme for AMP is increased, i.e., the addition of glucose 1-phosphate, P_i, F^-, divalent cations, or spermine, and a low temperature, and conversion of the enzyme to the *a* form. Conversely, it was weakened under the conditions for the opposite effect, i.e., the addition of glucose, a high temperature, and conversion of the enzyme to the *b* form. On the other hand, the extrinsic Cotton effects induced from binding of dAMP, GMP, and IMP are affected by the second ligands in an inverse manner to the case of AMP, although the effects are not so large. Substrates and protamine have been shown to have little effect on the affinities of the enzyme for many AMP analogues including the above nucleotides (Mott and Bieber, 1970; Black and Wang, 1968).

Since AMP differs from dAMP, GMP, and IMP in 2′-hydroxyl and 6-amino groups, it is possible that a new hydrogen bonding and/or an ionic bonding between these groups of the AMP and the enzyme are formed to cause its conformational change from the low affinity form to the high affinity one for the nucleotide, whereas no such bondings can be formed with the other nucleotides. An increase of numbers of the bonding between the AMP and the enzyme will cause (1) the more rigid configuration between the nucleotide and the enzyme restricting the perturbation of the AMP molecule in the allosteric site, and (2) a slight change in the configuration between the adenine base of the AMP and the aromatic group of the enzyme, thus, increasing the rotational strength of the extrinsic Cotton effects. On the other hand, as the other nucleotides do not have those groups in their structure to be linked with the enzyme, the affinities of phosphorylase *b* for these nucleotides, as well as the rotational strengths of the extrinsic Cotton effects by their binding, would not be altered by the additional factors.

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Ribose Recognition by Ribonuclease T_1: Difference Spectral Binding Studies with Guanosine and Deoxyguanosine†

Frederick G. Walz, Jr.

ABSTRACT: The binding of ribonuclease T_1 with guanosine (Guo) and deoxyguanosine (dGuo) was studied in experiments employing ultraviolet difference spectroscopy in the pH range 3–9 at 0.2 M ionic strength and 25 °C. Similar experiments were also conducted with γ-carboxymethyl-glutamate-58 ribonuclease T_1 at pH 5.0. At most pH values the characteristic difference spectrum and association constant were obtained. The binding constant for dGuo was ~550 M^{-1} and did not significantly vary in the pH range 3.5–9.0. The binding constant for Guo increased from pH 3.5 to 5.0, was constant between pH 5.0 and 7.0 (~3200 M^{-1}), and decreased at higher pH values. The binding of Guo and dGuo with ribonuclease T_1 could also be distinguished in terms of the wavelength for maximal difference absorbance, λ_{max}, between pH 5.0 and 7.0. At higher and lower pH values, λ_{max} for Guo approached that found fr dGuo. On the other hand, the value of the binding constant (~6500 M^{-1}) and the nature of the difference spectra for Guo and dGuo binding with γ-carboxymethyl-glutamate-58-ribonuclease T_1 at pH 5.0 were identical. These results suggest that the discrete interaction of the Guo 2′-hydroxyl group with ribonuclease T_1 involves the γ-carboxylate of glutamate-58 and an imidazolium group at the active site.

It is recognized that RNase[1] T_1 (EC 3.1.4.8) catalyzes RNA depolymerization via a two-step process including: (1) *Transesterification* in which the phosphoester bond between a guanosine 3′-P residue and the 5′ oxygen of the adjacent nucleoside group is cleaved with concomitant formation of a guanosine 2′,3′-P residue, and (2) *hydrolysis* in which the guanosine 2′,3′-P residue is specifically hydrolyzed to its 3′-monophosphate product (Takahashi et al., 1970). The simple fact that RNase T_1 polynucleotide substrates require a guanosine 2′-hydroxyl group for endonucleolytic cleavage immediately suggests the primary importance of the enzyme's interaction with this group in substrate recognition and catalysis. In this regard, a mechanism for the RNase T_1 catalyzed transesterification has been proposed (Takahashi, 1970a) which suggests, in part, that the substrate Guo 2′-hydroxyl

† From the Department of Chemistry, Kent State University, Kent, Ohio 44242. *Received March 12, 1976.* This work was supported by National Science Foundation Grant BMS76-01420.

[1] Abbreviations used are: RNase, ribonuclease; γ-CM-Glu-58-RNase T_1, RNase T_1 having a γ-carboxymethyl group on Glutamate 58; nucleoside designations follow the recommendation of the IUPAC-IUB commission as reported in *Biochemistry 9,* 4025 (1970); Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; uv, ultraviolet; NMR, nuclear magnetic resonance.