Cobalt(II) and Nickel(II) Complexes of Phosphoglucomutase†

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ABSTRACT: Properties of the cobalt(II) and nickel(II) complexes of rabbit muscle phosphoglucomutase are compared to those of the magnesium(II) complex. In terms of activity, pH–V_{max} profile and effect of temperature on V_{max}, the nickel(II) complex is similar to the magnesium(II) complex, while the cobalt(II) complex is different. A difference was also found in the shape of pH–V_{max} profiles for the cobalt(II) enzyme at 0 and 30°; the difference is caused by a change in the effect of temperature on enzymic activity with changing pH. By contrast, a similarity was observed in the shape of the corresponding plots for the nickel(II) enzyme at 0 and 30°. The contants for dissociation of cobalt(II) and of nickel(II) fromt heir respective active-site complexes were measured by a kinetic procedure; they are about 10^{-9} (pH 8.0) and 10^{-8} (pH 7.5), respectively, at 30°. An activity titration of "metal-free" phosphoglucomutase with cobalt(II) indicates a one to one stoichiometry, and at 28° yields a titration curve with a relatively sharp break. By contrast, at 0° the titration curve does not break sharply, although binding of cobalt(II) to the active site is not markedly decreased at the lower temperature; an even more dramatic change in titration with decreasing temperature was found for nickel(II). Apparently metals such as cobalt(II) and nickel(II) can be partitioned between the active site and ancillary binding sites, and with decreasing temperature the partitioning becomes more favorable relative to the latter types of sites. However, the spectrum of the 1:1 nickel(II) complex should reflect the environment of nickel(II) at the metal activating site, which in turn should reflect the environment of the normal activator, magnesium(II), at the same site.

Several properties of the Mg^{2+}, Mn^{2+}, Co^{2+}, and Zn^{2+} complexes of rabbit muscle phosphoglucomutase have been reported previously (Ray, 1969). The present paper describes a similar group of properties for the Ni^{2+} complex and provides additional data on the Co^{2+} complex. Primary emphasis is placed on those properties that have proved useful in evaluating the spectral studies described in the accompanying paper (Ray and Multani, 1972).

Experimental Section

Materials. Bovine serum albumin (crystalline) was obtained from Pentex; glucose-6-P dehydrogenase from Boehringer; NADP from Sigma; ethylenediaminetetraproponic acid from Eastern Chemicals, and nitrilotriacetic acid from Geigy; the latter material was crystallized from water before use.

Phosphoglucomutase, phospho form, was prepared by a scaled-up modification of the previously published procedure (Ray and Koshland, 1962). (Details will be given elsewhere but will be furnished on request prior to publication.) An absorbance of 7.7 at 278 mμ for a 1% solution (Najjar, 1955) and a molecular weight of 62,000 (Filmer and Koshland, 1963) were used in calculations of enzyme concentration. Activity is expressed in International Units and most of the enzyme used had an activity of greater than 800 IU/mg. "Metal-free" enzyme was prepared as described previously (Ray and Mildvan, 1970).

Enzymatic Assays. The following assay procedure was used routinely to assess the activity of the enzyme samples used in these studies and involves the Mg^{2+} form of the enzyme: a 0.98-ml aliquot of a stock solution of 4 mM glucose-1-P, 25 μM glucose-1,6-P_2, 0.2 mM NADP, 1 mM EDTA, 2 mM Mg^{2+}, and 50 mM Tris-HCl (pH 7.6) was placed in a 1 × 0.4 cm cuvet and brought to 30°. (Without Mg^{2+} the stock solution is stable in the frozen state.) A suspension of glucose-6-P dehydrogenase (5 mg/ml in 3.3 M $(NH_4)_2SO_4$) was diluted 1 to 5 with cold water and 0.01 ml of the dilute dehydrogenase was added to the cuvet just prior to the assay. The enzyme to be assayed was diluted to about 2 IU/ml in a solution 2.0 mM in Mg^{2+}, 1.0 mM in EDTA, 100 mM in imidazole, and 50 mM in Tris-HCl (pH 7.6), which contained 0.15 mg/ml of serum albumin. The solution was prepared daily from crystalline albumin and two stock solutions; one contained magnesium chloride and the other the remaining ingredients. After standing at least 5 min at room temperature, 0.01 ml of the diluted enzyme was added to the assay mixture in the cuvet and the change in absorbance at 340 mμ was followed for several minutes by using a Cary 15 spectrophotometer. Activity was calculated from the slope of the linear portion of the absorbance *vs.* time plot (an initial lag of about 20 sec was observed with the concentration of dehydrogenase used) by assuming that 1 mole of NADPH (ϵ_{340} 6.1) was produced for every mole of glucose-6-P formed by the phosphoglucomutase reaction. Agreement between this assay and the assay procedure described below was excellent.

Najjar's colorimetric assay (Najjar, 1955) based on the conversion of acid-labile to acid-stable phosphate and modified by Peck and Ray (1972) was used to measure the effect of temperature on the activity of the Mg^{2+} enzyme, except that the diluted enzyme was held at the assay temperature for about 10 min before initiating the assay. Similar assays were also conducted at pH 8.0, but with twice the concentration of glucose-1-P and glucose-1,6-P_2.

The effect of temperature and pH on the activities of the Mn^{2+}, Co^{2+}, and Ni^{2+}. enzymes was measured by means of the following assay. A 0.020-ml aliquot of diluted enzyme (see below) was added to 0.5 ml of 10 mM glucose-1-P, 25 μM

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glucose-1,6-P_2, 5 mM EDTA, and 20 mM Tris at the desired pH and temperature (10 mM maleate was included in the temperature study with the Ni^{2+} enzyme at pH 6.75). The reaction was quenched with 0.1 ml of 0.5 N hydrochloric acid; before determining the amount of glucose-6-P formed, the solution was neutralized with 0.1 ml of a solution prepared from equal volumes of 1 N sodium hydroxide and 1 N Tris (pH 7.5). In some cases the neutralized enzyme solution was centrifuged at this point. An aliquot of 0.010–0.050 ml of the neutralized solution was added to 0.7 ml of 0.1 M Tris (pH 7.5) and 30°, which contained 0.2 mM NADP and 0.25 μg of glucose-6-P dehydrogenase. The total optical density change produced at 340 mμ, which was proportional to the glucose-6-P present, was measured with a Cary 15 spectrophotometer equipped with a 0–0.1 OD slide-wire.

Enzyme dilutions were made as follows; Mn^{2+}–enzyme: an initial dilution, which contained about 1 mg/ml of protein in 10 mM Mn^{2+}–10.5 mM NTA[1]–20 mM Tris (pH 7.5), was allowed to stand several hours (usually overnight) before further dilution in the same buffer; Co^{2+}–enzyme: as above except that the initial dilution contained about 0.05 mg/ml of protein in 10 mM Co^{2+}–10.2 mM NTA–20 mM Tris (pH 7.5); Ni^{2+}–enzyme: a solution of metal-free enzyme was diluted with 1 mM Ni^{2+}–25 mM Tris-HCl (pH 7.5) to 3 mg/ml and allowed to stand several hours. Further dilutions of Ni^{2+}–enzyme of up to 100-fold were made in the same solution not more than 20 sec before use. Initial enzyme dilutions were kept at room temperature until used. The following time intervals were employed in these assays: Mn^{2+}–enzyme, 90 sec; Co^{2+}–enzyme, 30 sec; Ni^{2+}–enzyme, 20 sec.

Binding of Co^{2+} and Ni^{2+} to Phosphoglucomutase. To measure Co^{2+} binding to the enzyme, the concentration of free Co^{2+} in a Co^{2+}–NTA buffer (see above section) was varied by changing the [Co^{2+}]:[NTA] ratio in the presence of 20 mM Tris-HCl (pH 8.0) at 30°. A 0.1-ml aliquot of enzyme equilibrated in this buffer was added to 0.4 ml of assay solution; final concentrations were 0.5 mM glucose-1-P, 5 μM glucose-1,6-P_2, 10 mM EDTA, and 20 mM Tris-HCl (pH 8.5); 3-min assay intervals at 30° were employed. The assay was quenched with 0.5 ml of 2 N sulfuric acid and the decrease in acid-labile phosphate measured by the Bartlett procedure (Bartlett, 1959) as was described previously (Ray and Roscelli, 1964). The same solutions and procedure were used to assess Co^{2+} binding at 0°, except that the preassay equilibration was conducted at the lower temperature and the enzyme was assayed in a 30° water bath maintained in a cold room.

To measure Ni^{2+} binding, the concentration of Ni^{2+} to which the enzyme was exposed prior to the assay was varied by means of a Ni^{2+}–EDTP buffer, 20 mM in Tris-HCl (pH 7.5) and 30°. Metal-free enzyme was used and enzyme concentrations were maintained at 0.1 mg/ml or greater in the equilibration step. After exposure to the Ni^{2+} buffer for 2 min, aliquots of 0.001 ml were assayed by addition to 0.5 ml of a solution containing 3 mM glucose-1-P, 5 μM glucose diphosphate, 20 mM Tris-HCl, and 10 mM EDTA (pH 7.5). An assay interval of 20 min at 30° was used and color developed as above. (During the assay interval all of the Ni^{2+} dissociates from the enzyme under these conditions.)[2]

Activity Titrations at 0 and 28°. The activity elicited by addition of Co^{2+} or Ni^{2+} to a sample of metal-free enzyme was followed by removing aliquots of 0.001–0.005 ml and assaying in the presence of excess EDTA, as was done in the study of Co^{2+} and Ni^{2+} binding, above; however, here the assay concentrations of glucose-1-P and glucose-1,6-P_2 were 10 mM and 10 μM, respectively and the assay interval was 15 min in both cases. Titrations performed at 0° were conducted in an ice bath in a 2° cold room and those at 28° in a water bath in a warm room at the same temperature because of the small volumes of enzyme used in the assay. For the Co^{2+}–enzyme both titration and assay were conducted at the same temperature; for the Ni^{2+}–enzyme assays were conducted at 28° in both cases, even though one of the titrations was conducted at 0°.

Results

Activity of the Nickel(II)–Phosphoglucomutase Complex. The Ni^{2+}–phosphoglucomutase complex formed by direct combination of Ni^{2+} (0.1–3 mM) and moderately concentrated solutions (3 mg/ml) of metal-free enzyme was stable at room temperature for up to 2 days (pH 7.5). However, activity losses could be detected within a few minutes if a further 30-fold dilution was made *in the same solution* and activity losses of more than 50% were observed for 200-fold dilutions within 10 min. Since the Ni^{2+} in the original solution was in large excess over enzyme, the further dilution can scarcely inactivate by increasing the Ni^{2+} to enzyme ratio. Several alternative dilution techniques were tested, and although some were partially successful, in no case could maximal activity be maintained for 10 min after a further 30-fold dilution of the enzyme. For this reason, microliter volumes of the stock solutions of phosphoglucomutase and Ni^{2+} were used in the assay procedure (see Experimental Section) so that as little dilution as possible was required prior to the assay; when dilution was required, each dilution was used to initiate an assay immediately after preparation from the stock solution. By using this procedure an assay response was obtained that was linear in enzyme concentration. In addition, in short-term assays, product–time plots were linear if no more than 20% of the substrate were converted to product. In long-term assays (18 min) essentially all the Ni^{2+} dissociated from the enzyme because of the excess EDTA ($\tau_{1/2} \sim 3.5$ min);[2] hence the latter assay was similar to the all-or-none assays used previously for other metal complexes of phosphoglucomutase (Ray, 1969). Under both conditions, plots (not shown) of product produced *vs.* enzyme used in the assay were linear for dilutions of the stock solution as large as 100-fold. Assay errors were about $\pm 3\%$.

Binding of Nickel(II) and Cobalt(II) to Phosphoglucomutase. After exposing samples of phosphoglucomutase to a range of free Co^{2+} or Ni^{2+} concentrations, which were established with Co^{2+}–NTA, Ni^{2+}–EDTP, or Ni^{2+}–histidine buffers (Reilley, 1961), the fraction of the enzyme to which metal was bound at the metal-activating site was assessed by a procedure analogous to that used previously for Mn^{2+} and Zn^{2+} binding (Ray, 1969), *viz.*, by assaying the enzyme in the presence of excess EDTA. Double-reciprocal plots (not shown) of the activity elicited under these conditions and the concentration of free Co^{2+} to which the enzyme was exposed *prior to the assay* were linear and had slope to intercept ratios corresponding to a dissociation constant for the Co^{2+}–enzyme complex of about 1×10^{-9} M at pH 8 and 30°. (Free Co^{2+} was calculated by using $K_{d(Co \cdot NTA)} = 2 \times 10^{-11}$

[1] The following abbreviations are used: NTA, nitrilotriacetic acid; EDTP, ethylenediaminetetrapropionic acid. The subscript T is used to denote all species of the indicated moiety.

[2] D. S. Goodin and W. J. Ray, Jr., unpublished results.

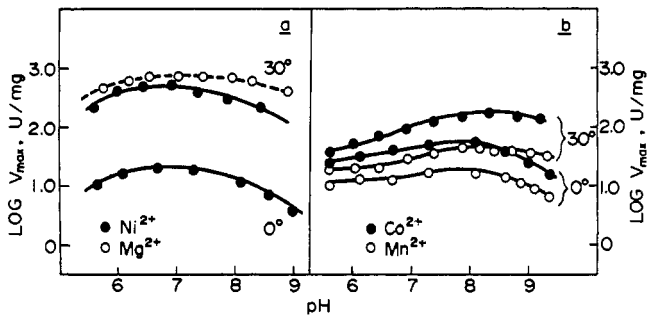

FIGURE 1: Effect of pH and temperature on the activities of the nickel(II), cobalt(II), and maganese(II) forms of phosphoglucomutase. Conditions are described in the Experimental Section. (a) Log V_{max} *vs.* pH for the Ni^{2+}–enzyme at 0 and 30°; data for the Mg^{2+}–enzyme (Ray, 1969) are given for comparison, ○. (b) Log V_{max} *vs.* pH for the Co^{2+}, ●, and Mn^{2+}–enzymes, ○, at 0 and 30°.

M and $K_{a(NTA)} = 2 \times 10^{-10}$ M (Sillen and Martell, 1964).)[3] The same results were obtained by keeping $[Co]_T$ constant and varying $[NTA]_T$ or by keeping $[NTA]_T$ constant and varying $[Co]_T$; hence ternary complexes involving enzyme metal and chelating agent apparently are unimportant in this system. (Similar conclusions have been reached for other metal complexes of phosphoglucomutase, see Ray, 1969.) Plots for Ni^{2+} analogous to those described for Co^{2+} tended to be concave upward. The curvature may have been caused by the peculiar instability of the Ni^{2+}–phosphoglucomutase complex (see previous section). However, buffering at 10^{-8} M Ni^{2+} with either EDTP or histidine produced half-maximal activity in the subsequent assay, *viz.*, half the activity produced by treatment with mM Ni^{2+}. Hence, K_d for the Ni^{2+}–enzyme complex is in the range of 10^{-8} M.

The binding of Co^{2+} at 0° was similar examined (data not shown). However, no values of $K_{d(Co \cdot NTA)}$ have been published for this temperature. If the above value (for 20°) is used together with $K_{a(NTA)} = 10^{-9.7}$ at 0° (Sillen and Martell, 1969), the apparent K_d for the Co^{2+}–enzyme complex is 2×10^{-9}; *viz.*, the enzyme changes its affinity for Co^{2+} to a similar extent as does NTA from 30 to 0°.

The Effect of pH on the Activities of the Nickel(II), Cobalt(II), and Manganese(II) Forms of Phosphoglucomutase at 0 and 30°. Figure 1a shows the effect of pH on the activity of the Ni^{2+}–enzyme at 0 and 30°; Figure 1b shows the results of a similar study of the Mn^{2+} and Co^{2+} forms of the enzyme. The recorded pH's refer to the actual pH at the indicated temperature after addition of the enzyme aliquot. Linearity of the assay with time was checked at extremes of both pH and temperature; the use of the assay intervals indicated in the Experimental Section insured that the measured velocity was within 10% of v_0, as deduced from product time plots (not shown), although longer assay intervals at the higher temperatures in the neutral pH range could have been employed. The substrate concentration was saturating at the extremes of both pH and temperature as indicated by the lack of change in measured velocity when both glucose-1-P and glucose-1,6-P_2 were decreased by 50%; hence, V_{max} values were measured at all points.

The V_{max} *vs.* pH profile for the Ni^{2+}–enzyme is similar at 0 and 30° (Figure 1a). Hence, the apparent value of V_{max} measured at the pH of maximum activity, 6.75, should be very close to the value of the pH-independent parameter. The V_{max} *vs.* pH profile of the Mg^{2+}–enzyme at 30° (Ray, 1969) is also shown in Figure 1a for comparison. The profiles for the Ni^{2+} and Mg^{2+} forms are nearly congruent at 30° and low pH but diverge somewhat at higher pH's. By contrast the shapes of the V_{max} *vs.* pH profiles for the Co^{2+}– and Mn^{2+}–enzymes are rather different at 0 and 30°. At 30° the plots are similar to those reported previously (Ray, 1969) except that the present study was extended to somewhat higher pH's and the downward drift in the high pH range is thus more prominent; hence, the previous analysis of pH effects on these forms of the enzyme in terms of a strictly sigmoidal V_{max} *vs.* pH plot was an oversimplification of the overall effect. This is especially obvious in the plots at 0°. Because of the odd shapes of these curves no further attempts were made to analyze them on a quantitative basis.

Effect of Temperature on the Activity of the Magnesium(II) and Nickel(II) Forms of Phosphoglucomutase. The effect of temperature on the activity of the Ni^{2+}–enzyme at pH 6.75 and of the Mg^{2+}–enzyme at pH 7.5 and 8.0 was measured between 0 and 40° in the manner described in the Experimental Section. No attempt was made to compensate for temperature-induced changes in the pH of the Tris or Tris–maleate buffers (which were adjusted to pH at room temperature), since the change in V_{max} with pH (Figure 1) is sufficiently small to make such compensation unnecessary. Controls at 0, 20, and 40° similar to those described above were used to verify that V_{max} had indeed been measured. Semilog plots of V_{max} *vs.* $1/T$ for the Ni^{2+}–enzyme at pH 6.75 and the Mg^{2+}–enzyme at pH 7.5 are shown in Figure 2; the plot for the Mg^{2+}–enzyme at pH 8.0 was essentially the same as the plot shown. Both curves shown in Figure 2 have the same shape; they differ only by an arbitrary vertical displacement of 0.15 log unit.[4] Hence, under optimal conditions the Ni^{2+}–enzyme is about 70% as active as the Mg^{2+}–enzyme. Also shown in this figure are the activities of the Co^{2+}– and Mn^{2+}–enzyme at 0 and 30° (pH 7.5).

Titration of Phosphoglucomutase with Cobalt(II) and Nickel(II) at 0 and 28° as Followed by Activity Regain. As a control for experiments in which the effect of temperature on the spectra of the Co^{2+} and Ni^{2+} complexes of the enzyme were examined (see Ray and Multani, 1972), activity titrations with both metals were compared at 0 and 28°. These titrations were followed by assaying aliquots from the titration mixture in the presence of a *large excess* of EDTA, and using the regain in activity as a measure of metal binding to the activating site. Note that the presence of substrate in the assay does not alter the fraction of enzyme with metal bound at the activating site, since any free metal ion present during the titration is bound to EDTA during initiation of the assay. Moreover, migration of metal ions from an ancillary site to the active site is also prevented by the excess EDTA present.

At 28°, slightly less than complete activity regain is found at a 1:1 ratio of Co^{2+} to enzyme (Figure 3a) and at 0° (Figure 3a) the titration curve shows noticeable curvature in this region. The absence of a sharp break in the curve is not caused by incomplete binding of Co^{2+} by the enzyme in the titration

[3] The values of $K_{d(Co \cdot NTA)}$ and $K_{a(NTA)}$ used here are those given for 20° and $\mu = 0.1$; although a higher temperature and a lower ionic strength were used to establish free Co^{2+} concentrations, the error involved in using the values given for the stated conditions is relatively small.

[4] Harshman and Najjar (1965) report a linear Arrhenius plot for the Mg^{2+}–enzyme; however, their results are not inconsistent with a shallow curve of the type shown.

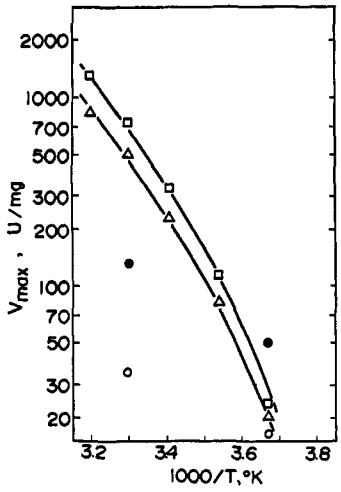

FIGURE 2: Effect of temperature on the activities of the nickel(II) and magnesium(II) forms of phosphoglucomutase. Activities for the various forms of phosphoglucomutase were determined under conditions where both metal ion and substrate were saturating; for the Mg^{2+} and Ni^{2+} forms the pH was in the flat portion of the velocity profile: 6.75 for Ni^{2+} and 7.5 for Mg^{2+} (see Results). For comparison the activities of the Co^{2+}- and Mn^{2+}-enzymes at pH 7.5, ● and ○, respectively, are shown at 0 and 30° (data from Figure 1b). The activity scale is logarithmic.

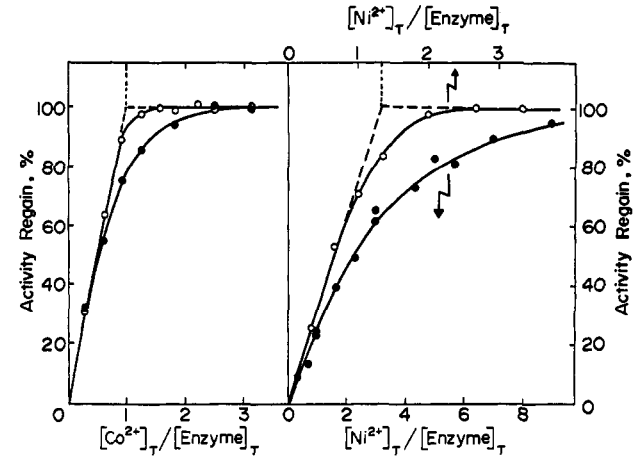

FIGURE 3: Titration of phosphoglucomutase with cobalt(II) and nickel(II) at 0 and 28°. (a) Cobalt(II) titration. To solution of metal-free phosphoglucomutase, 2.4 mg/ml (0.0385 mM) in 20 mM Tris (pH 7.5) at 28° (○) or 0° (●), were added aliquots of Co^{2+}; 1.5 min after each addition samples of 0.001 ml were removed and assayed. For details, see the Experimental Section. The activity regain in percent is plotted against the metal:enzyme ratio. (b) Nickel(II) titration. The titration was conducted as described for Co^{2+} in part a (see also the Experimental Section); ○, and *upper* scale, 28°; ●, and *lower* scale, two titrations at 0°.

mixture, as is indicated by binding studies described above (K_d is about 10^{-9} M for dissociation of Co^{2+} from the active site at 30° and the value is not substantially changed at 0°; the enzyme concentration used in the titration was 30 μM) and by the similarity of the results in Figure 3a and those of analogous spectral titrations (see Ray and Multani, 1972) which were conducted at an enzyme concentration more than 10-fold higher than that used here. An alternative rationale is thus indicated: that binding at ancillary sites is small but detectable at 28° and becomes relatively more important at 0°. The presence of a single ancillary site with about 0.1 the affinity for Co^{2+} as the active site would produce a titration curve similar to that obtained at 0°.

The results of analogous titrations with Ni^{2+} are shown in Figure 3b. Because of technical problems related to low activity of the Ni^{2+}–enzyme at 0°, the enzyme titrated at 0° was assayed at 28°; however, the entire procedure was conducted so that no temperature change could occur until the enzyme was in the presence of a large excess of EDTA. The curve obtained with Ni^{2+} at 28° (Figure 3b) is similar to that obtained at 0° with Co^{2+} (Figure 3a). Since Ni^{2+} is bound at the active site nearly as tightly as Co^{2+}, extraneous binding sites are again indicated. Although the constant for dissociation of Ni^{2+} from the active site at 0° was not measured, it seems unlikely that the dissociation constant increases by many orders of magnitude in going from 30 to 0°—especially since the analogous constant for Co^{2+} apparently does not change markedly over this temperature range—as would be required to explain the shape of 0° titration curve (Figure 3b) in terms inefficient binding of Ni^{2+} by the enzyme. Moreover, the activity elicited at a Ni^{2+}:enzyme ratio of 1:1 did not change as enzyme concentration was increased or decreased twofold from that used in the titration. Hence, the plot at 0° indicates that in the case of Ni^{2+}, extraneous binding at numerous sites competes successfully with binding at the active site. However, Ni^{2+} binding at ancillary sites does not significantly decrease the activity of the enzyme, at least when it is assayed in the presence of EDTA, since the per cent activity elicited in the titration at 0° was calculated on the basis of the maximal activity produced in a titration at 30°.

Discussion

It is usually conceeded that *equilibrium* binding of metals to enzymes cannot be determined by steady-state kinetic measurements. However, previous work with phosphoglucomutase has shown that such equilibria can indeed be assessed kinetically if (a) excess EDTA is employed in the steady state reaction so that all free metal ion is eliminated prior to initiation of the assay[5] and (b) if the metal ion dissociates from the enzyme sufficiently slowly under these conditions to permit meaningful activity measurements (Ray and Roscelli, 1966a,b; Ray, 1967, 1969). Had not kinetic procedures been employed in the present study the results would have been difficult if not impossible to unravel. In fact, a kinetic procedure such as that used here is preferable to static procedures such as equilibrium dialysis when the metal ion is bound tightly or when binding at ancillary sites also is involved.

The present results indicate that both Ni^{2+} and Co^{2+} are bound sufficiently tightly to phosphoglucomutase so that spectral titrations of the metal binding site with either of these metals is feasible at enzyme concentrations in excess of 10^{-4} M. In addition, in both cases active site binding predominates over ancillary binding at a 1:1 stoichiometry at room temperature (see below). Although a proof for mutually exclusive binding of Mg^{2+}, Co^{2+}, and Ni^{2+} at the metal activating site has not been obtained because of technical difficulties, previous studies do show mutually exclusive binding by Mg^{2+}, Zn^{2+}, and Mn^{2+} (Ray, 1969) and it seems likely that this type of binding also extends to Co^{2+} and Ni^{2+}.

[5] In the present study most assays were conducted by adding the equilibrated enzyme–metal mixture directly to an EDTA–substrate mixture (see Experimental Section) because of the convenience of this procedure; however, results were always spot-checked by employing a rapid, sequential addition of EDTA and substrate.

In such a case these metals deserve consideration as spectral probes of the Mg^{2+} binding site of the enzyme.

In terms of activity characteristics, such as V_{max} value, pH profile, and effect of temperature on V_{max}, the Ni^{2+}–enzyme proved to be similar to the Mg^{2+}–enzyme while the Co^{2+}–enzyme is markedly different. The similarity in pH profiles obtained with the Ni^{2+}–enzyme at 0 and 30° suggests that the identity of the rate determining step or steps for this enzyme complex may well be pH independent in the neutral range. By contrast, the difference in shape of the pH profiles obtained with the Co^{2+}–enzyme at 0 and 30° suggests that the identity rate determining steps for the Co^{2+} form of the enzyme may be different at low and high pH (see Figure 1). However, the complexity of the curves discourages further speculation. That the Co^{2+} complex is not unique in its odd pH profile is indicated by the similar pH profile for the Mn^{2+} complex, both at 0 and 30°, which is also shown in Figure 1b.

The correspondence in the effect of temperature on V_{max} for the Mg^{2+}– and Ni^{2+}–enzymes in the flat portions of their pH profiles suggests but certainly does not prove that the two complexes involve similar metal ion binding. Such a suggestion is based both on the rather generally accepted feeling that active sites of enzymes are critically structured entities and the substantial evidence that the metal binding site is intimately related to if not part of the active site of phosphoglucomutase. Such evidence includes (a) ultraviolet difference spectroscopy (Peck and Ray, 1969), (b) measurements of water proton relaxation rates induced by the Mn^{2+}–enzyme (Ray and Mildvan, 1970), evaluation of electron paramagnetic resonance spectra of the Mn^{2+}–enzyme (Reed and Ray, 1971), and approximation of the metal of methyl group distance for methyl phosphonate bound at the anionic binding site of the Mn^{2+}–enzyme by means of nuclear magnetic resonance techniques (W. J. Ray, Jr., and A. S. Mildvan, manuscript in preparation).

The lack of correspondence in temperature effect on V_{max} for the Mg^{2+}– and Co^{2+}–enzymes actually is rather uninformative. Possibly the latter complex does indeed involve differences in binding at the metal binding site. However, it is also possible that differences in the chemical character of the two metals causes a change in the identity of the rate-controlling step or steps in the catalytic process. Such a metal-specific change has been suggested in the alkaline phosphatase system (Lazdunski and Lazdunski, 1969). If the rate-controlling step is different for the Co^{2+}– and Mg^{2+}–enzymes a difference in the effect of temperature on V_{max} would be expected, even if metal binding were identical. In any case, caution must be exercised in using Co^{2+} as a probe of the Mg^{2+} binding site in this enzyme system.

Unfortunately, the Co^{2+}–enzyme is somewhat better behaved than the Ni^{2+}–enzyme, both with respect to stability in dilute solution (see Results section) and to preferential binding at the metal activating site. Thus in the case of the 1:1 Co^{2+} complex, binding is nearly exclusively to the active site at 30°, and while ancillary binding becomes more important at 0°, active site binding still predominates by severalfold at the lower temperature. By contrast, although active site binding in the 1:1 Ni^{2+} complex predominates by severalfold at 30° it represents only a minor fraction of the total at 0°. However, because of the relative spectral characteristics of both Co^{2+} and Ni^{2+} bound predominately at the active site as opposed to these metals bound predominately at ancillary sites, spectra for the 1:1 Co^{2+} and Ni^{2+} complexes at 27° should constitute a reasonable representation of the spectrum of the active-site metals (Ray and Multani, 1972).

The change in binding specificity from preferential binding at the active site at 28° to preferential binding at ancillary sites at 0° was unexpected and is difficult to rationalize in terms of simple temperature effects on binding constants. Thus, if the binding ratio at the two types of sites is to change 30-fold, for example, with a 28° change in temperature a *difference* in $\Delta H°$ for binding at the two types of sites of some 20 kcal would be required. Alternatively, $\Delta H°$ for one or both binding processes could be temperature dependent, possibly because of temperature-induced conformation changes in the enzyme system.

In any case, the present results indicate that considerable caution should be exercised in interpreting the effect of temperature on properties of metal–enzyme complexes in terms of the properties of a single species unless a unique complex is verified at all temperatures studied. For example, the effect of temperature on the water proton relaxation rate induced by the Mn^{2+}–phosphoglucomutase complex has been reported, although stoichiometric binding of Mn^{2+} at the active site of the enzyme was investigated only at 30° (Ray and Mildvan, 1970). Fortunately, subsequent titrations at 0° also showed preferential binding of Mn^{2+} at the active site, although evidence was obtained for an increased importance of metal binding at ancillary sites (W. J. Ray, Jr., and A. S. Mildvan, unpublished results) as was found in the present study for Co^{2+} and especially for Ni^{2+}.

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