Phosphorylation Mechanism of Nucleoside Diphosphate Kinase: ^{31}P-Nuclear Magnetic Resonance Studies†

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Received May 24, 1995; Revised Manuscript Received July 24, 1995⊗

ABSTRACT: The phosphorylation mechanism of *Dictyostelium discoideum* nucleoside diphosphate (NDP) kinase was investigated by NMR. ^{31}P chemical shifts were measured on both native and denatured enzyme. In the enzymatically phosphorylated enzyme denatured by 9 M urea or 7 M guanidine hydrochloride, the NDP kinase phosphohistidine signal appeared between the signals of Nδ and Nε free monophosphohistidines used as reference compounds and added to the sample. A signal with the same intermediate position was also observed in the pronase digest of the alkaline-denatured phosphorylated enzyme. However, when phosphohistidines of the phosphorylated synthetic peptide pGlu-His-Gly were taken as references, the NDP kinase and the Nδ peptide phosphohistidine signals were shown to be identical, providing evidence that phosphorylation occurs on the Nδ of the active site histidine residue. Moreover, the rate of hydrolysis of the histidine-bound phosphate is in agreement with a modification at the Nδ position. Phosphorylation of the NDP kinase by phosphoramidate provided a result similar to that of the enzymatic phosphorylation. In both cases, phosphorylation could not be detected on any amino acid other than histidine. Particularly, no phosphoserine residue was observed.

NDP kinases[1] (EC 2.7.4.6) are enzymes which catalyse the transfer of the γ-phosphate from nucleoside 5′-triphosphates to nucleoside 5′-diphosphates, following a ping-pong mechanism:

$$XTP + E \leftrightarrow XDP + E{-}P$$

$$E{-}P + YDP \leftrightarrow E + YTP$$

The phosphate transfer operates via the phosphorylation of a histidine during the catalytic cycle (Parks & Agarwal, 1973). NDP kinases display no specificity for the nucleotide base and can phosphorylate deoxynucleotides as well as their oxy derivatives.

The first three-dimensional structure of a NDP kinase to be reported was that of a point mutant of the enzyme from the slime mold *Dictyostelium discoideum* (Dumas et al., 1992). The hexameric protein is made of two trimers, with three 2-fold axis and one 3-fold axis. The structure of each 17 kDa subunit contains a central antiparallel β-sheet connected by α-helices. Each subunit carries one active site as demonstrated by the X-ray structure of the binary complexes with ADP and thymidine diphosphate (dTDP) (Morera et al., 1994; Cherfils et al., 1994). The nucleotide binding mode is different from that observed in other phosphotransferases (Schulz, 1992). In particular, there is no interaction of the nucleotide with the protein backbone. The base makes only nonpolar contacts with the enzyme while the ribose and the pyrophosphate make hydrogen bonds with several side chains. A Mg^{2+} ion bridges the α-phosphate to the β-phosphate.

The three-dimensional structure of the NDP kinases from *Drosophila melanogaster* and from the prokaryote *Myxococcus xanthus* have also been reported (Chiadmi et al., 1993; Williams et al., 1993). The subunits' fold is remarkably similar in all cases, although the quaternary structure is different; like the *Dictyostelium* enzyme, the *Drosophila* NDP kinase is a hexamer while the bacterial enzyme is a tetramer. Even so, the complex between the enzyme from *Myxococcus* and ADP shows a very similar mode of nucleotide binding to each subunit as compared to the *Dictyostelium* enzyme (Williams et al., 1993). Thus, it seems likely that the current structural data available on the nucleotide binding mode of the NDP kinases from *Dictyostelium* and *Myxococcus* provide a general framework for the nucleotide binding mode of other NDP kinases, including the human NDP kinases A and B (Gilles et al., 1991), encoded by the genes *nm23*-H1 (Steeg et al., 1988) and *nm23*-H2 (Stahl et al., 1991), respectively.

Histidine 122 was shown to be the catalytic histidine in the *Dictyostelium* NDP kinase (Wallet, 1992; Dumas et al., 1992). The structure of the enzyme complexed with ADP and dTDP suggests a transfer of the nucleotide γ-phosphate to the Nδ of the catalytic histidine (Morera et al., 1994;

† This work was supported by funds from the Institut Pasteur and the Centre National de la Recherche Scientifique (URA 1129), the Association de la Recherche contre le SIDA (Grant 61216), and the Association de la Recherche contre le Cancer (Grant 6438). A.B. was a recipient of an EC fellowship.

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⊗ Abstract published in *Advance ACS Abstracts,* September 1, 1995.

[1] Abbreviations: NDP, nucleoside diphosphate; PEP, phosphoenolpyruvate; Pi, inorganic phosphate; Gu HCl, guanidine hydrochloride; pGlu, pyroglutamic acid; Nδ and Nε correspond to the two nitrogen positions in histidine which are named respectively N1 or τ and N3 or π in some publications.

0006-2960/95/0434-12445$09.00/0

Cherfils et al., 1994). Indeed, the His 122 side chain is in a position to be phosphorylated on its Nδ. The Nε should therefore be protonated and form a hydrogen bond with the Glu 133 side chain. This model was recently supported by site-directed mutagenesis studies of the active site where the replacement of Glu 133 by a lysine totally abolished the enzymatic activity, although residue 133 is away from ADP in the binding site (Tepper et al., 1994).

In this paper, we describe the investigation of *Dictyostelium* NDP kinase phosphorylation using ^{31}P-NMR. We demonstrate that autophosphorylation occurs at the Nδ position of the active site histidine. Phosphorylation could not be detected on any other amino acid, including serine residue, in agreement with the recent data of Bonimaar et al. (1994). This is in contrast to previous reports of serine phosphorylation in NDP kinase from *Myxococcus* (Muñoz-Dorado et al., 1993), from human (MacDonald et al., 1993), and from the rat mucosa mast cells (Hemmerich et al., 1992). Finally, the chemical phosphorylation by phosphoramidate is shown to produce the same compound as autophosphorylation.

EXPERIMENTAL PROCEDURES

Chemicals. Only analytical grade chemicals were used throughout this work. Pronase E (protease type XXV) and pGlu-His-Gly acetate salt were from Sigma; ATP, PEP, and pyruvate kinase were from Boehringer, Manheim. Urea and Gu HCl were ultrapure reagents from Schwartz-Mann.

Phosphoenzyme Preparation. Dictyostelium NDP kinase was overexpressed in *Escherichia coli* (Lacombe et al., 1990) and purified as described (Wallet et al., 1990), except that the enzyme was dialyzed against 25 mM ammonium bicarbonate pH 8 and freeze-dried before use. The protein concentration was calculated from UV absorbtion spectrum at 280 nm using an extinction coefficient of 0.6 $(mg/mL)^{-1}\cdot cm^{-1}$ (Wallet, 1992). All enzyme preparations were checked for phosphorylation stoichiometry (Lascu et al., 1993).

Enzymatic Phosphorylation. The reaction was performed *in situ* in the NMR tube using an ATP regenerating system according to the following scheme:

$$\text{NDP kinase} + \text{ATP} \leftrightarrow \text{NDP kinase}{-}\text{P} + \text{ADP} \quad (1)$$

$$\text{ADP} + \text{PEP} \xrightarrow{\text{pyruvate kinase}} \text{ATP} + \text{pyruvate} + \text{Pi} \quad (2)$$

Hydrolysis of the phosphohistidine occurred as a side reaction:

$$\text{NDP kinase}{-}\text{P} \rightarrow \text{NDP kinase} + \text{Pi} \quad (3)$$

NDP kinase, 1 mM based on a 16.8 kDa subunit molecular mass, was incubated at 25 °C with 100 μM ATP, 5 mM PEP, 5 mM $MgCl_2$, and 6.5 units of pyruvate kinase in 650 μL of 50 mM Tris-HCl pH 8.1, 90/10 D_2O/H_2O (v/v) buffer. The appearance of the phosphorylated enzyme was followed by ^{31}P-NMR.

Chemical Phosphorylation. Dipotassium phosphoramidate was synthesized as described by Wei and Matthews (1991). Phosphorylation was accomplished by the dissolving of 16 mg of NDP kinase and 20 mg of dipotassium phosphoramidate in 650 μL of 50 mM Tris-HCl pH 8.1, 90/10 D_2O/H_2O (v/v) buffer. The reaction was performed at 25 °C and monitored by ^{31}P-NMR.

Denaturation. The denaturation of the phosphorylated NDP kinase was performed by rapid addition of urea or Gu HCl to the reaction mixture when the intensity of the phosphorylenzyme signal became significant. Final concentrations of the denaturing agents were 9 and 7 M, respectively.

Pronase Digestion. An alkaline denaturation of the phosphorylated NDP kinase was performed before pronase digestion in order to make proteolysis more efficient. The pH of the enzyme solution was increased to 11.5 with 1 N NaOH and readjusted to 8.1 with 1 N HCl. These pH modifications caused a partial precipitation of the enzyme. Pronase E (10% w/w) was added to the sample, and the mixture was gently shaken for 45 min at 25 °C. The digest was centrifuged for 5 min at 10000*g*, and NMR spectra of the supernatant were recorded.

Preparation of Reference Phosphohistidine Compounds. Free mono- and diphosphohistidines were synthesized as described by Gassner et al. (1977). Histidine HCl (5 mg) and dipotassium phosphoramidate (11 mg) were dissolved in 0.7 mL of 50 mM Tris-HCl solution. The pH was adjusted to 8.1, D_2O was added to 10% (v/v), and formation of the three derivatives was followed by ^{31}P-NMR.

Peptide pGlu-His-Gly (3 mg) was phosphorylated on the Nδ and Nε positions of the histidine residue by addition of phosphoramidate (20 mg) to the peptide solution (0.7 mL of 50 mM Tris-HCl pH 8.1, 10% D_2O buffer). Phosphorylation was monitored by ^{31}P-NMR.

Phosphorus NMR Experiments. NMR spectra were recorded on a Varian Unity 500 spectrometer equipped with Sun Sparc computers. Spectra were recorded at 202 MHz using a 5 mm direct broad band (50−202 MHz) probe. A 70° excitation pulse was used with a repetition delay of 2 s, a spectral width of 20 000 Hz, and 25 600 data points. Spectra were referenced to 85% H_3PO_4 (external). No apodization function was applied when the line widths were measured; otherwise, a line broadening of 5 or 10 Hz was used. Due to the high molecular weight of the enzyme and the subsequent line broadening, no proton decoupling was performed as proton phosphorus coupling constants are small.

RESULTS AND DISCUSSION

Analysis of the Enzymatically Phosphorylated Enzyme. The NDP kinase phospho derivative is labile on the time scale of the NMR experiment (Bominaar et al., 1994). In addition, a low ATP/NDP kinase ratio was used in order to reduce the intensity of the ATP signals in the spectra and to minimize their interference with other signals. For these reasons, it was necessary to use an ATP regenerating system to accumulate an amount of phospho derivative sufficient to be observed with a reasonable number of scans. As shown in Figure 1A, the ^{31}P-NMR spectrum of the phosphorylated enzyme in the native state is comprised of three major peaks. The sharp peak at 0.13 ppm was present at the beginning of the phosphorylation reaction, and its intensity decreased with time. It corresponds to phosphoenolpyruvate. The two peaks at 3.47 and −2.74 ppm appeared during the course of the reaction. The chemical shift of the first one, with a line width of 6 Hz, is consistent with that of inorganic phosphate at the pH used in the experiment. The second peak at −2.74 ppm, with a line width of 96 Hz, corresponds to the phosphoryl enzyme. Its width, compatible with that expected

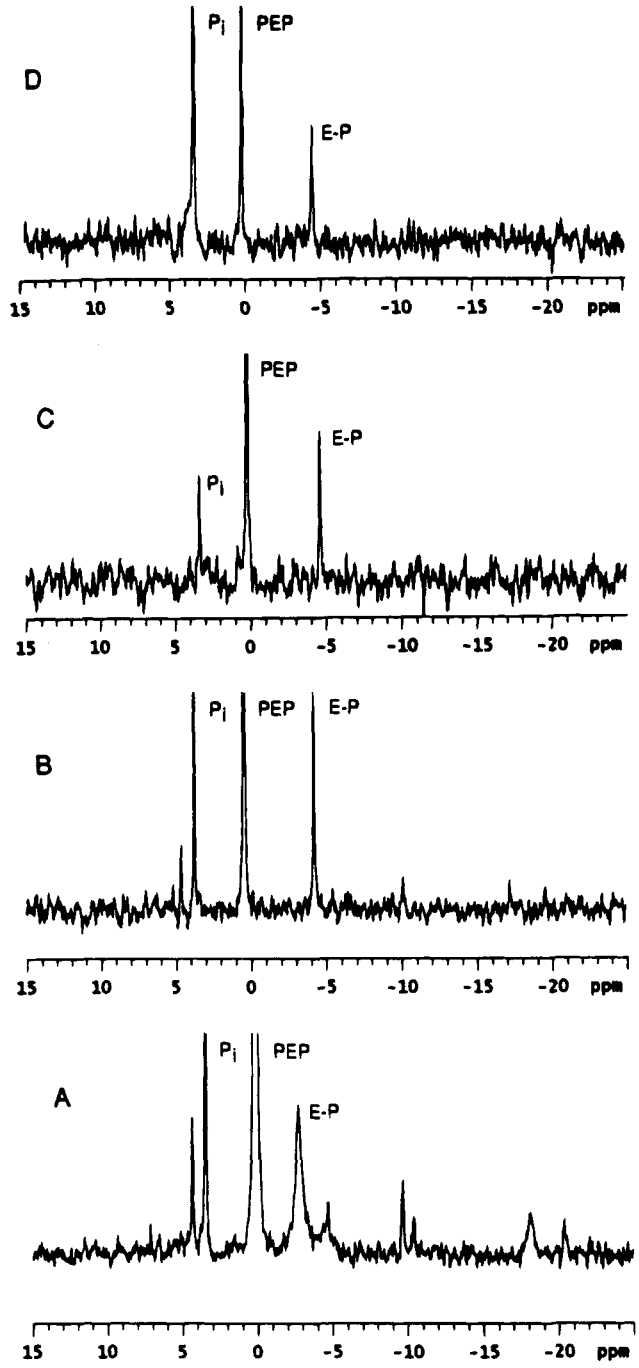

FIGURE 1: Spectra at 25 °C of enzymatically phosphorylated NDP kinase prepared as described in Experimental Procedures. (A) Native enzyme, 2048 scans; (B) 9 M urea-denatured enzyme; (C) 7 M Gu HCl-denatured enzyme; (D) pronase digest of the alkaline-denatured enzyme. Spectra in B−D were recorded with 256 scans. Pi, inorganic phosphate; PEP, phosphoenolpyruvate; E-P, phosphoenzyme signals.

for a protein of such a size, and the fact that it only appeared when all reagents were present in the sample agree with this assignment. The small peak at 4.34 ppm is due to a contaminant present in the preparation used in the experiment described in Figure 1A. It was not found in other experiments (see Figure 1C,D and Figure 6). Other minor peaks with chemical shifts ranging between −4.7 and −20.3 ppm correspond to ATP signals either complexed with Mg^{2+} or not complexed (Gorenstein, 1984).

The denaturation of the phosphoprotein was performed to identify the resonance of the protein-bound phosphate. Inactivation and unfolding of *Dictyostelium* NDP kinase in urea were previously investigated by measurement of the effect of this denaturing agent on the fluorescence, the ellipticity, and the activity of the enzyme, demonstrating that denaturation occurred between 5 and 6 M urea (Lascu et al., 1993). Urea was added to the phosphorylated sample up to 9 M, and spectra were recorded as soon as it was solubilized. Denaturation resulted in a large upfield shift of the peak at −2.74 to −4.20 ppm ($\Delta\delta = -1.46$ ppm) and in a marked reduction of its line width from 96 to 7 Hz. No other new sharp peak appeared. Other signals in the spectrum were only slightly downfield-shifted by the denaturing agent (Figure 1B). The signal observed at −4.20 ppm was assigned to phosphohistidine on the basis of its chemical shift. The free phosphoamino acid signals indeed appear between −4.5 and −4.9 ppm for the Nδ−P signal and between −3.6 and −4.0 ppm for the Nϵ−P signal, depending on the histidine protonation state (Gassner et al., 1977). It should be noted that the values given above were obtained by adding 0.8 ppm to the values reported by Gassner et al., to take into account the use of a superconducting magnet instead of an electromagnet (Gadian, 1982). The deprotonated form of phosphoserine gives a signal between 4.6 and 4.8 ppm (Brauer & Sykes, 1984). The fact that no peak was detected in this chemical shift region indicates that the phosphoserine signal, if any, would be in the background. The corresponding serine phosphorylation level would then be less than 1%, in agreement with recent results of Bominaar et al. (1994), who demonstrated that less than 0.2% of NDP kinase subunits were autophosphorylated on non-histidine residues. This result is in contrast to the serine phosphorylation which was described for the NDP kinase from *Myxococcus* (Muñoz-Dorado et al., 1993), from human (MacDonald et al., 1993), and from rat mucosa mast cells (Hemmerich et al., 1992) enzymes.

The characterization of phosphorylated positions on histidine residues is commonly carried out through direct comparison of the protein phosphoderivative chemical shift in its unfolded form with chemical shifts reported in the litterature for the phosphohistidine amino acid. However, the signals corresponding to both Nδ and Nϵ phosphohistidines are separated by less than 1 ppm, and many factors such as the small variations of pH that occur during the phosphorylation reaction, the composition and the concentration of the buffer in the sample, and the presence of urea or of other agents can induce significant shifts that could invalidate their identification. A mixture (10 μL) of the phosphohistidine reference compounds, prepared from free histidine as described in Experimental Procedures, was therefore directly added to the denatured enzyme sample, and spectra were immediately recorded. The two mono- and diphosphohistidine signals could be easily identified through comparison with a spectrum of the three compounds recorded independently (data not presented). As shown Figure 2A, the two resonances of Nδ and Nϵ phosphohistidines were respectively observed at −4.66 and −3.66 ppm. The NDP kinase phosphohistidine signal, with a chemical shift of −4.19 ppm, does not however correspond to either of them, preventing the identification of the phosphorylated nitrogen atom. Peaks at −3.28 and −4.31 ppm correspond to the Nϵ−P and Nδ−P of the diphosphohistidine. This result suggested either that some residual secondary structure persisted in 9 M urea or alternatively that a sequential effect occurred.

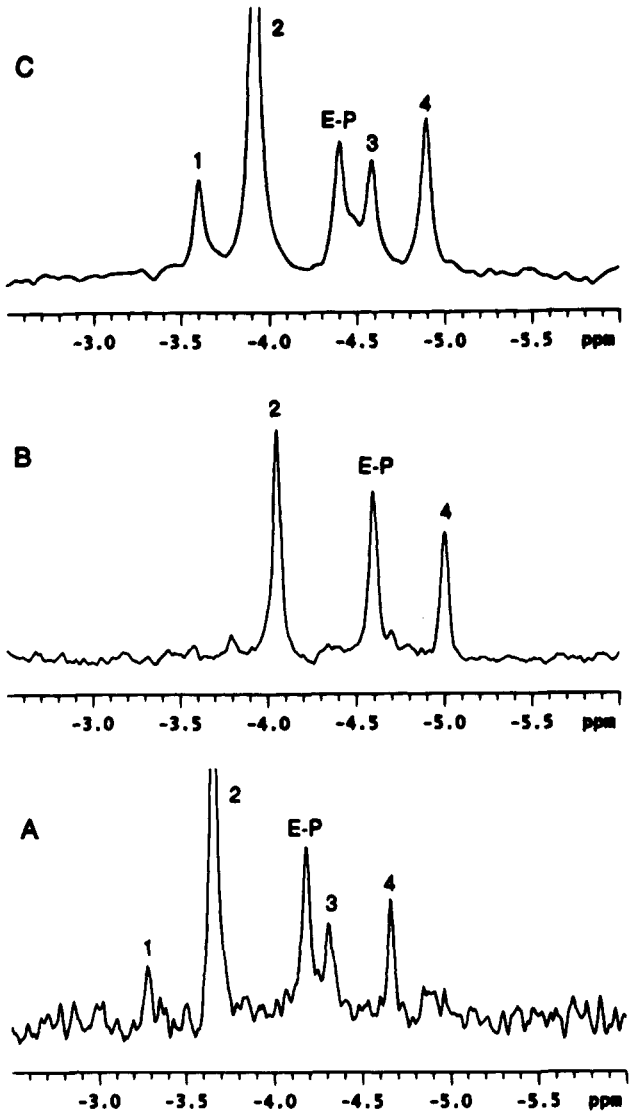

FIGURE 2: Spectra at 25 °C of the denatured phosphorylated NDP kinase after addition of 10 μL of the reference free phosphohistidines reaction mixture. (A) 9 M urea denaturation, (B) 7 M Gu HCl denaturation, (C) pronase digestion after alkaline denaturation. All spectra were recorded with 256 scans. Free phosphohistidine signals: 1, Nϵ−P diphosphohistidine; 2, Nϵ−P monophosphohistidine; 3, Nδ−P diphosphohistidine; 4, Nδ−P monophosphohistidine. E-P, phosphoenzyme signal.

Thus, in a second set of experiments, Gu HCl, a stronger denaturating agent, was used at 7 M final concentration. The phosphohistidine signal of the denatured enzyme was observed at −4.60 ppm (Figure 1C). Like urea, guanidine induced a large upfield shift of the phosphoenzyme signal ($\Delta\delta$ = −1.88 ppm), concomitant with a reduction of its line width to 6 Hz. The Nδ and Nϵ phosphohistidine reference compounds added to the NDP kinase sample gave signals at −5.01 and −4.05 ppm, respectively. Again, the peak corresponding to the phosphoenzyme had an intermediate position between these two signals (Figure 2B). It is worth noting that the relative height of the four reference signals in the spectra depends on the time elapsed since the beginning of the phosphorylation reaction. Indeed, it has been demonstrated that phosphoramidate first reacts with the Nδ position of free histidine to generate Nδ phosphohistidine. Within a few minutes, the monophospho Nδ compound is then further phosphorylated and transformed to diphosphohistidine. As the diphospho compound hydrolyzes preferentially at the Nδ position, the net reaction is a conversion of the Nδ to the Nϵ phosphohistidine which thus accumulates in the sample (Hultquist et al., 1966).

In order to eliminate the possibility that some residual structure persisted in 9 M urea or 7 M Gu HCl, phosphorylated NDP kinase was denatured in alkali and further digested with pronase E, a nonspecific proteolytic enzyme. The digestion was followed by the recording of successive spectra of the digest. The phosphohistidine signal was shifted from −2.74 to −4.40 ppm and its line width reduced to 7 Hz (Figure 1D). Nevertheless, signals from the Nδ and Nϵ phosphohistidine reference compound mixture added to the sample appeared respectively at −4.90 and −3.61 ppm (Figure 2C), once again on both sides of the NDP kinase signal. The digestion conditions were however sufficient to cleave the region containing the catalytic histidine into small peptides, since the enzyme phosphohistidine signal did not move with time.

We concluded that the shifts observed between the NDP kinase phosphohistidine and the reference compounds signals in the last three experiments were not due to a residual structure still present after urea or guanidine denaturation, or after pronase proteolytic cleavage. Since the amino acid composition in the catalytic His 122 neighborhood contained no aromatic and no titratable group, sequential effects were unlikely. Therefore, the probable explanation for these shifts was that the compounds used as references in this as in other studies (Gassner et al.,1977; Fujitaki et al., 1981; Williams et al., 1985; Thrall et al., 1993) were inadequate.

As compared with a histidine residue encompassed in a peptide, the histidine amino acid has two additional charges at the pH used, both at its N and C termini. Therefore, the Nδ−P and Nϵ−P signals of mono and diphosphohistidines might have slightly different chemical shifts in the free charged amino acid and in a peptide histidine residue. In order to test this hypothesis, the peptide pGlu-His-Gly was phosphorylated with phosphoramidate as described in Experimental Procedures. The phosphorylation was followed by the recording of successive spectra of the phosphopeptide which were compared with those of free phosphohistidines. The spectrum of a mixture of the phosphopeptide and of free phosphohistidines (Figure 3A) shows that the signals of the two phosphorylated compounds are not superimposable. Whereas monophosphohistidines Nϵ−P signals for both compounds have an identical chemical shift (−4.01 ppm), the peptide monophosphohistidine Nδ−P signal (−4.41 ppm) is downfield-shifted with respect to the free histidine monophosphohistidine Nδ−P signal (−4.95 ppm). The peptide Nϵ−P and Nδ−P diphosphohistidine signals are respectively upfield- (0.06 pm) and downfield-shifted (0.41 ppm) as compared to the free histidine diphosphohistidine signals. In constrast, the ^{31}P signal in the digest of NDP kinase appeared to correspond exactly to the Nδ phosphohistidine signal of the phosphorylated peptide. We confirmed this by recording the spectrum of the digest after addition to the sample of 10 μL of both phosphorylated peptide and phosphorylated histidine (Figure 3B).

These results show unambiguously that the NDP kinase catalytic His 122 is phosphorylated at the Nδ position. The rate of hydrolysis of the histidine-bound phosphate in the enzyme is also consistent with the fact that the phosphorylation occurs on the histidine Nδ site rather than on the Nϵ

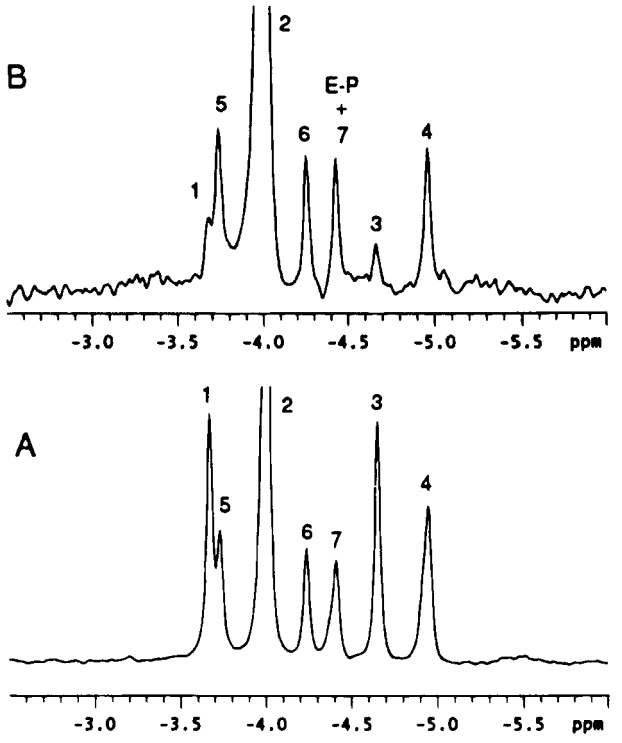

FIGURE 3: (A) Spectrum at 25 °C and pH 8.1 of a mixture of phosphorylated pGlu-His-Gly peptide and of phosphorylated free histidine. (B) Pronase digest of alkaline-denatured phosphorylated NDP kinase after addition of 10 μL of both phosphorylated pGlu-His-Gly peptide and free histidine reaction mixtures. Free phosphohistidine signals: 1, Nϵ−P diphosphohistidine; 2, Nϵ−P monophosphohistidine; 3, Nδ−P diphosphohistidine; 4, Nδ−P monophosphohistidine. Peptide pGlu-His-Gly phosphohistidine signals: 5, Nϵ−P diphosphohistidine; 2, Nϵ−P monophosphohistidine; 6, Nδ−P diphosphohistidine; 7, Nδ−P monophosphohistidine. E-P, phosphoenzyme signal. Spectra were recorded with 512 scans.

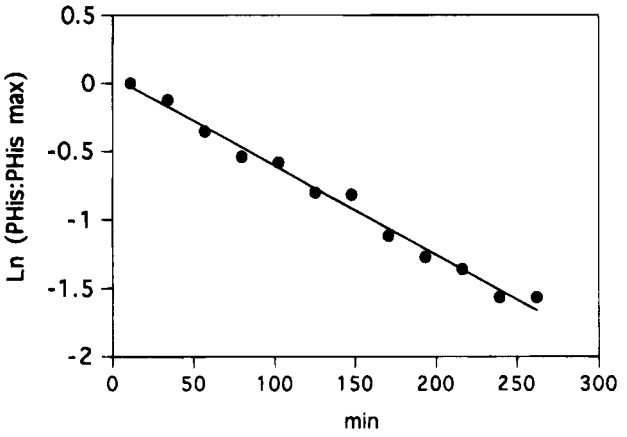

FIGURE 4: Hydrolysis kinetics of histidine-bound phosphate. The peak height of the phosphorylated histidine was measured on successive spectra recorded with 512 scans.

site. Indeed, in contrast to Nδ phosphohistidine which has been shown to hydrolyze rapidly, Nϵ phosphohistidine is stable for several days (Hultquist et al., 1966). The stability of the phosphohistidine in the native enzyme was assessed by the recording of successive spectra of 256 transients each, as soon as all of the PEP had been consumed. The half-life of the phospho derivative could be evaluated as 106 min at pH 8.1 and 25 °C (Figure 4), in good agreement with previous data (Bominaar et al., 1994).

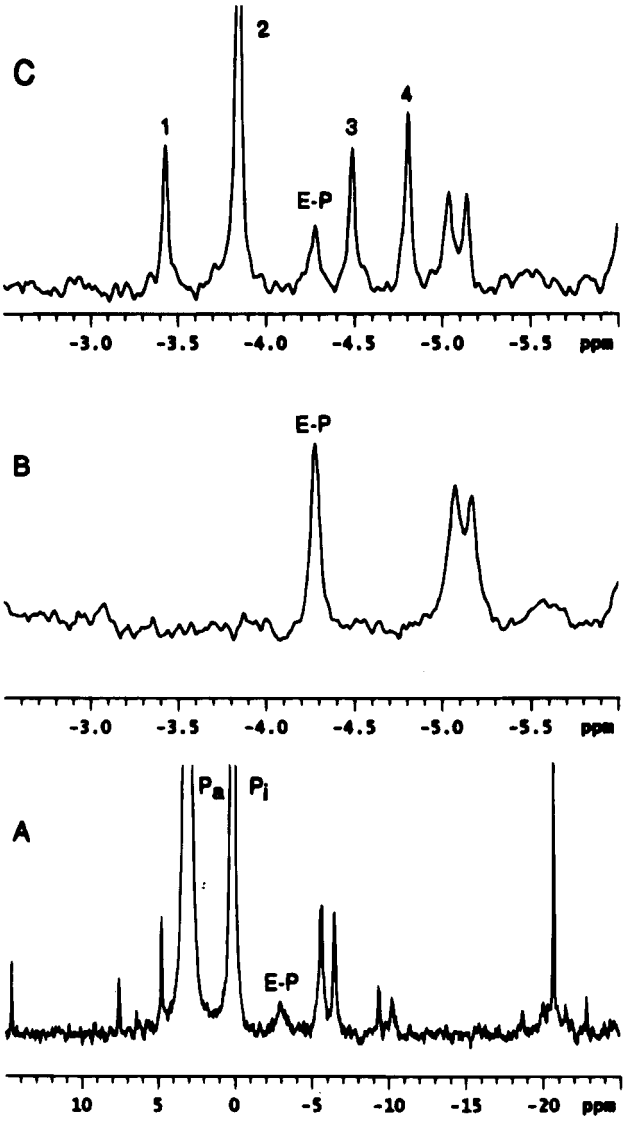

FIGURE 5: Spectra at 25 °C of chemically phosphorylated NDP kinase. (A) native enzyme, 2048 scans; (B) 9 M urea-denatured enzyme, 756 scans; (C) 9 M urea-denatured enzyme after addition of 10 μL of the reference free phosphohistidines reaction mixture, 756 scans. Pi, inorganic phosphate; Pa, phosphoramidate; E-P, phosphoenzyme signals. Free phosphohistidine signals: 1, Nϵ−P diphosphohistidine; 2, Nϵ−P monophosphohistidine; 3, Nδ−P diphosphohistidine; 4, Nδ−P monophosphohistidine.

Analysis of the Chemically Phosphorylated Enzyme. The phosphorylation of NDP kinase by phosphoramidate was monitored by the recording of successive spectra as a function of time. As seen in Figure 5A, a large peak appeared at about −3 ppm in the native enzyme. Due to their line width and their appearance or disappearance kinetics, the other signals in the spectra could be unambiguously attributed to Pi (0.13 ppm), phosphoramidate (3.03 ppm), and byproducts of phosphoramidate. After denaturation of the sample with 9 M urea, the peak at −3 ppm was shifted to −4.25 ppm and its line width decreased to 6 Hz (Figure 5B). All other sharp peaks were already present in the native state or could be assigned to the phosphoramidate byproducts. As shown in Figure 5C, the phosphohistidine reference compounds added to the denatured sample gave signals at −4.81 ppm (Nδ−P) and −3.84 ppm (Nϵ−P), respectively. The spectrum was similar to those obtained with the enzymatically phosphorylated NDP kinase. Shifts

of 0.1−0.2 ppm observed in the native as well as in the denatured state are due to the presence of phosphoramidate in the sample and to the small pH variations induced by this reagent during the phosphorylation reaction. Thus, phosphoramidate appears to produce exactly the same phospho derivative as the enzymatic phosphorylation. The catalytic histidine 122 is the only residue phosphorylated by the chemical, and the modification occurs on its Nδ position. However, the *Dictyostelium* NDP kinase contains three histidine residues, His 55, 59, and 122, two of which, His 55 and 122, are located in the active site, approximately orthogonal, and in van der Waals contact (Morera et al., 1994). Phosphorylation of the catalytic histidine 122 on its Nδ position, and of that histidine only, in crystals of the *Dictyostelium* and the *Drosophila* enzymes treated with phosphoramidate was also recently described (Morera et al., 1995).

Our results unambiguously demonstrate that enzymatic phosphorylation of *Dictyostelium* NDP kinase occurs on the Nδ position of the catalytic histidine 122. They validate the mechanism for catalysis suggested from structural data on complexes formed with ADP and dTDP (Morera et al., 1994; Cherfils et al., 1994) and by site-directed mutagenesis of active site residues (Tepper et al., 1994). This mechanism likely provides a general framework for the nucleotide binding mode of other NDP kinases since the active site residues are strictly conserved.

Determination of the target site on the imidazole of histidine-phosphorylated proteins is usually done by ^{31}P-NMR since structural data of the phosphorylated forms are only available for HPr (Van Nuland et al., 1995) and succinyl-Co A synthetase (Wolodko et al., 1994). Proteins are denatured or enzymatically digested, and the chemical shifts of the phosphohistidine signals are compared with the chemical shifts of free phosphohistidines independently determined on a sample of the reference compounds or merely reported in the literature. In this paper, we have provided evidence that the use of free phosphohistidine chemical shifts as references is unsuitable for determining the phosphorylated position of a histidine in a polypeptide. The reference compound should be a phosphohistidine included in a small peptide in order to have the histidine N and C termini blocked, and the peptide should not contain any other aromatic residue or titratable group which could induce a sequential effect on the chemical shifts. It is also essential to have an internal reference. Differences between the protein and the reference compounds buffers, especially the small pH variations that inevitably occur during the phosphorylation reaction, may induce shifts of the signals sufficient to lead to misinterpretations. Identification of other modified residues in proteins by direct comparison of their chemical shifts with those of the free modified amino acids should undoubtedly also be handled with care.

ACKNOWLEDGMENT

Thanks are due to Dr. G. Déléris for help in synthesis of phosphoramidate. We are grateful to Dr. J. Janin for data communication on the X-ray structures of the phosphoramidate-phosphorylated NDP kinases from *Dictyostelium* and *Drosophila* and for helpful discussions and to Dr. M. Goldberg for his pertinent suggestions.

REFERENCES

Bominaar, A. A., Tepper, A. D., & Véron, M. (1994) *FEBS Lett. 353*, 5−8.

Brauer, M., & Sykes, B. D. (1984) *Methods Enzymol. 107*, 37−81.

Cherfils, J., Morera, S., Lascu, I., Véron, M., & Janin, J. (1994) *Biochemistry 33*, 9062−9069.

Chiadmi, M., Morera, S., Lascu, I., Dumas, C., Le Bras, G., Véron, M., & Janin, J. (1993) *Structure 1*, 283−293.

Dumas, C., Lascu, I., Morera, S., Glaser, P., Fourme, R., Wallet, V., Lacombe, M. L., & Janin, J. (1992) *EMBO J. 11*, 3203−3208.

Engel, M., Issinger, O.-G., Lascu, I., Seib, T., Dooley, S., Zang, K. D., & Welter, S. (1994) *Biochem. Biophys. Res. Commun. 199*, 1041−1048.

Fujitaki, J. M., Fung, G., Oh, E. U., & Smith, R. A. (1981) *Biochemistry 20*, 3658−3664.

Gadian, D. G. (1982) *Nuclear magnetic resonance and its application to living systems*, p 102, Clarendon Press, Oxford.

Gassner, M., Stehlick, D., Schrecker, O., Hengstebberg, W., Maureer, W., & Ruterjans, J. (1977) *Eur. J. Biochem. 75*, 287−296.

Gilles, A. M., Presecan, E., Vonica, A., & Lascu, I. (1991) *J. Biol. Chem. 266*, 8784−8789.

Gorenstein, D. G. (1984) *Phosphorus-31 NMR*, pp 57−103, Academic Press, Inc., London.

Hemmerich, S., & Pecht, I. (1992) *Biochemistry 31*, 4580−4587.

Hultquist, D. E., Moyer, R. W., & Boyer, P. D. (1966) *Biochemistry 5*, 322−331.

Lacombe, M. L., Wallet, V., Troll, H., & Véron, M. (1990) *J. Biol. Chem. 265*, 10012−10018.

Lascu, I., Deville-Bonne, D., Glaser, P., & Véron, M. (1993) *J. Biol. Chem. 268*, 20268−20275.

MacDonald, N. J., De La Rosa, A., Benedict, M. A., Freije, J. M. P., Krutsch, H., & Steeg, P. S. (1993) *J. Biol. Chem. 268*, 25780−25789.

Morera, S., Lascu, I., Dumas, C., Le Bras, G., Briozzo, P., Véron, M., & Janin, J. (1994) *Biochemistry 33*, 459−467.

Morera, S., et al. (1995) *Biochemistry* (in press).

Muñoz-Dorado, J., Almaula, N., Inouye, S., & Inouye, M. (1993) *J. Bacteriol. 175*, 1176−1181.

Parks, R. E., & Agarwal, R. P. (1973) *Enzymes 8*, 307−334.

Rajagopal, P., Waygood E. B., & Klevit, R. E. (1994) *Biochemistry 33*, 15271−15282.

Schultz, G. E. (1992) *Curr. Opin. Struct. Biol. 2*, 61−67.

Stahl, J. A., Leone, A., Rosengard, A. M., Porter, L., King, C. R., & Steeg, P. (1991) *Cancer Res. 51*, 445−449.

Steeg, P. S., Bevilacqua, G., Kopper, L., Thorgeirsson, U. P., Talmadge, J. E., Liotta, L. A., & Sobel, M. E. (1988) *J. Natl. Cancer Inst. 80*, 200−204.

Tepper, A. D., Damman, H., Bominar, A., & Véron, M. (1994) *J. Biol. Chem. 269*, 32175−32180.

Thrall, S. H., Mehl, A. F., Carrol, L. J., & Dunaway-Mariano, D. (1993) *Biochemistry 32*, 1803−1809.

Van Nuland, N. A. J., Boelens, R., Scheek, R. M., & Robillard, G. T. (1995) *J. Biol. Mol. 246*, 180−193.

Wallet, V. (1992) *Etude de la Nucleoside Diphosphate Kinase de Dictyostelium*, Ph.D. Dissertation, University of Paris 7, Paris, France.

Wallet, V., Mutzel, R., Troll, H., Barzu, O., Wurster, B., Véron, M., & Lacombe, M. L. (1990) *J. Natl. Cancer Inst. 82*, 1199−1202.

Wei, Y.-F., & Matthews, H. R. (1991) *Methods Enzymol. 200*, 388−414.

Williams, R. L., Deena, A. O., Munoz-Dorado, J., Inouye, S., Inouye, M., & Arnold, E. (1993) *J. Biol. Mol. 234*, 1230−1247.

Williams, S. P., Sykes, B. D., & Bridger, W. A. (1985) *Biochemistry 24*, 5527−5531.

Wolodko, W. T., Fraser, M. E., James, M. N. G., & Bridger, W. A. (1994) *J. Biol. Chem. 269*, 10883−10890.

BI951168D