Compartmentation of Nucleotides in Corn Root Tips Studied by $^{31}$P-NMR and HPLC

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ABSTRACT

Corn (Zea mays L.) root tips were subjected to different conditions so that nucleotide levels varied over a wide range. Levels of nucleotides in corn root tips were measured using $^{31}$P nuclear magnetic resonance (NMR) spectroscopy and high performance liquid chromatography. Results indicate: (a) Similar amounts of NTP and sugar nucleotides were observed by in vivo NMR and in extracts. In contrast, a significant amount of NDP observed in root tip extracts was not detected by in vivo NMR. Thus, for a given sample, [NTP]/[NDP] ratios determined in vivo by $^{31}$P-NMR are always higher than ratios observed in extracts, deviating by ~4-fold at the highest ratios. The NMR-invisible pool of NDP appeared quite metabolically inert, barely changing in size as total cell NDP changed. We conclude that NDP in corn root tips is compartmented with respect to NMR visibility, and that it is the NMR-visible pool which responds dynamically to metabolic state. The NMR-invisible NDP could either be immobilized (and so have broad, undetectable NMR signals), or be complexed with species that cause the chemical shift of NDP to change (so it does not contribute to the NMR signal of free NDP), or both. (b) $^{31}$P-NMR cannot distinguish between bases (A, U, C, and G) of nucleotides. HPLC analysis of root tip extracts showed that the relative amount of each base in the NTP and NDP pools was quite constant in the different samples. (c) In extracts, for each of the nonadenylate nucleotides, [NTP]/[NDP] was linearly proportional to [ATP]/[ADP], indicating near equilibrium in the nucleoside diphosphokinase (NDPK) reaction. However, the apparent equilibrium constants for the phosphorylation of GDP and UDP by ATP were significantly lower than 1, the true equilibrium constant for the NDPK reaction. Thus, for a given sample, [ATP]/[ADP] ~ [CTP]/[CDP] ~ [UTP]/[UDP] ~ [GTP]/[GDP]. This result suggests that the different NDPs in corn root tips do not have equal access to NDPK.

NTP metabolism in plants has been most widely studied by enzymatic or chromatographic analysis of extracts, with attention almost exclusively focused on adenylates (17). More recently, $^{31}$P-NMR has been used to examine nucleotide metabolism in living plants (19, 20) and has generated one result potentially in conflict with extract studies: the [NTP]/[NDP] ratios in corn root tips observed in vivo by $^{31}$P-NMR (22) are significantly higher than [ATP]/[ADP] ratios determined in extracts (25, 26). This apparent discrepancy could result from any, or some combination, of four phenomena, which we address in this study. First, workers using in vivo NMR could simply have studied tissues in a different metabolic state than the tissues used in the extract studies; we examined this possibility by directly comparing root tip samples in vivo with extracts of the same samples. Second, contributions from nonadenylate nucleotides, which cannot be distinguished from ATP and ADP by $^{31}$P-NMR, could in principle make [NTP]/[NDP] > [ATP]/[ADP]; we therefore determined [ATP]/[ADP], [UTP]/[UDP], [CTP]/[CDP] and [GTP]/[GDP] in root tips extracts by HPLC. Third, breakdown of NTP to NDP during extraction would lead to lower [NTP]/[NDP] in extracts than in vivo; we tested this possibility by quantitation of NTP and NDP in vivo and in extracts. Finally, a significant proportion of certain nucleotides may be undetectable by in vivo $^{31}$P-NMR due to molecular interactions (e.g. with proteins). This could result either from interactions causing immobility, since high resolution NMR detects signals only from freely mobile species (21), or from interactions that cause large changes in chemical shifts, relative to free nucleotides, as occurs on binding of GDP to elongation factor Tu (31). Any such distinctions between nucleotides in vivo is lost on extraction. Compartmentation of nucleotides between immobilized and freely mobile pools was suggested in NMR studies of human blood platelets (4, 28), skeletal muscle (7), and rat kidney (6), from comparison of the amounts of nucleotides visible in vivo and in extracts. Such comparisons were made in this study, and our results indicate the existence of an NMR-invisible pool of NDP in vivo.

Compartmentation of nucleotides in cells can also be inferred from metabolite analysis of near-equilibrium reactions. The mass action ratio for a reaction known to operate near equilibrium in vivo is determined by analysis of the whole cell or tissue. Compartmentation of one or more substrates away from the particular enzyme is suggested when the mass action ratio differs significantly from the equilibrium constant. Using this approach, together with a comparison of tissues differing in mitochondrial content, Veech et al. (29) concluded that much of the ADP in cells is sequestered in mitochondria, and therefore inaccessible to cytoplasmic enzymes on the timescale of nucleotide turnover (seconds). Here we described an analogous study of the NDPK2 reaction, using HPLC to analyze root tip extracts. NDPK catalyzes the transfer of the

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2 Abbreviations: NDPK, nucleoside diphosphokinase; MDP, methylene diphosphonate; cNADP, $\beta$-NAD-2',3'-cyclic monophosphate.
terminal phosphate from NTP to NDP (14). In vivo, ATP produced via oxidative phosphorylation normally drives phosphorylation of NDPs produced by metabolism (18). The equilibrium constant of the NDPK reaction is approximately 1 (14). The activity of NDPK in many tissues is high relative to activities of other enzymes (1, 14), such that near equilibrium is anticipated (18). Our analysis suggests compartmentation of the different NDPs in corn root tips with respect to access to NDPK.

**MATERIALS AND METHODS**

**Plant Material**

Corn (*Zea mays* L.) hybrid Funk 4323 (Germain’s Seeds, Los Angeles, CA) was soaked in water for 24 h, then germinated in the dark for 36 to 48 h. Two g (~1000) 2 mm long root tips were excised on ice with a razor blade, rinsed and transferred to a 3 mL plastic syringe (~10 mm diameter) (the NMR sample tube). A capillary tube containing 0.5 M MDP in Tris (pH 8.9) was placed in the center of the sample tube. The sample was perfused as described previously (19). Perfusion conditions were varied to produce a wide range of NTP/NDP ratios in the different samples analyzed. NTP/NDP ratios in samples perfused rapidly (>15 mL/min) with an oxygen-bubbled solution were consistently greater than 20, while those for samples perfused slowly (7–8 mL/min) with an air saturated, 100 μM KCN solution were as low as 0.5.

**Nucleotide Extraction**

After NMR data accumulation, the syringe was drained and the tissue rapidly frozen by immersion in liquid nitrogen. A modified TCA in ether procedure was used for nucleotide extraction (30). This method has been described as the most reliable procedure for nucleotide extraction (15); we found this to be so, compared to extractions using perchloric acid, as we employed previously (22). The frozen sample was removed from the syringe and placed in a −20°C mortar with 60 μL of a frozen solution of 6.9 mM cNADP and 7.0 mM XMP, both from Sigma, St. Louis, MO. The sample was ground to a fine powder at −20°C, then transferred on dry ice to a fume hood. Nine mL of 0.6 M TCA/ether, chilled on dry ice, was added and the sample homogenized for 1 min. The slurry and solid residue were spun at 16,000g for 10 min at 4°C in a stainless steel tube with cap. The supernatant was saved and 2 mL TCA/ether added to the pellet, which was resuspended and recentrifuged. The two supernatants were pooled and extracted twice with equal volumes of water. The aqueous phases were pooled and washed four times with two volumes of ether to remove the TCA. The remaining water phase was vigorously bubbled with nitrogen gas to remove the ether. Samples were neutralized with KOH, frozen, and lyophilized. Samples were resuspended in 600 μL of extraction buffer (60 mM Mops, 2 mM EDTA, 15 mM MgCl₂, brought to pH 7.6 with Tris base) and transferred to a 5 mm NMR tube containing the MDP capillary reference. The high magnesium ion concentration was used to resolve the signal of the internal standard (cNADP) from the low field sugar nucleotide resonance (see peaks 5 and 6 in Fig. 1E).

**NMR Spectroscopy**

Fourier transform 31P-NMR spectra were obtained on a General Electric GN500 spectrometer operating at 202.5 MHz. Neither field frequency locking nor proton noise decoupling was used. Chemical shifts are referenced to external MDP, at 0 ppm. All spectra were obtained under nonsaturating conditions (pulse interval 5 s; pulse angle ~60°) so that peak intensity was proportional to concentration in the NMR tube (19). In vivo NMR spectra were acquired in 1 h blocks so that the stability of spectra over time could be established. Several blocks were added together to give spectra of sufficient signal/noise for nucleotide quantitation. Typical acquisition times are given in the legend to Figure 1. Spectra of extracts were acquired over ~5 h; no changes in extract spectra were observed over 24 h at room temperature. All NMR spectra peaks were integrated by cutting and weighing. NTP was determined from the area of the γ-NTP resonance, NDP from the β-NDP resonance (Fig. 1)(22). The higher free magnesium concentration in the extracts relative to in vivo (~1 mm) (19) resulted in less frequency separation between γNTP and βNDP, and resolution αNTP and αNDP (compare Fig. 1, B and C).

Nucleotides *in vivo* were quantitated by comparing their respective peak areas to the peak area of a 1 mM phosphate solution in a 3 mL syringe. Comparison was made using the signal from MDP (contained in a coaxial glass capillary in both samples) as a reference. This comparison gave nucleotide concentrations in the NMR detection volume. To convert concentrations to content (μmol/500 root tips), this concentration was multiplied first by the volume of 500 root tips (initially ~1.21 mL) and second by the growth factor (final/initial volume). Nucleotides in extracts were quantiated using the signal from the cNADP internal standard (620 nmol 31P/500 root tips). The contribution from endogenous NAD(P) to the cNADP signal was subtracted using a standard curve of NAD(P) peak height versus [NTP]/[NDP], determined from spectra of 21 root tip extracts, varying in [NTP]/[NDP], to which cNADP was not added. The area of the endogenous NAD(P) signal was ~20% of the cNADP signal (Fig. 1).

**HPLC**

After NMR analysis, the extract was purified using the tricolumn system described by Nieman *et al.* (11). HPLC was performed using the following equipment: 2 Altex model 100 pumps controlled by a Altex model 420 microprocessor; Hewlett Packard 1040A HPLC-[diode-array] detection system; Hewlett packard 85B computer and integration system; Rheodyne model 7120 injector with a 20 μL loop; Whatman Partispherf 5 SAX anion exchange cartridge column (250 × 4.6 mm); Whatman guard column of the same material; silica saturator pregauvuar column packed with 50 μm silica gel; Orion Research digital pH meter 611. The separating, guard, and pregauuar column were held at 30°C with water jackets. The buffer and gradient system were as described previously (12). Peaks were identified based on comparison of retention times and absorption spectra of authentic, pure nucleotide. Nucleotides were quantitated using the XMP internal standard. The ratio of peak areas, nucleotide/XMP, recorded at
RESULTS

Differences between NTP/NDP Ratios Observed in vivo and in Extracts Are Due to Pools of NMR-Invisible NDP

When a given corn root tip sample was examined in vivo by $^{31}$P-NMR, [NTP]/[NDP] was consistently higher than in the corresponding extract (Fig. 2). Further, the highest value of [NTP]/[NDP] (or [ATP]/[ADP] discussed below) in extracts reported here are similar in magnitude to the highest values reported by others (e.g. Refs. 25 and 26). Therefore, the differences in [NTP]/[NDP] between in vivo $^{31}$P-NMR and extract analyses must be due to changes during extraction; either conversion of NTP to NDP, or release of bound and NMR-invisible NDP, such that more NDP is detected in the extract. We distinguished between these two possibilities by determining the absolute amounts of NTP and NDP observed in vivo and in extracts by $^{31}$P-NMR (Figs. 3 and 4). No difference between the amount of NTP observed in vivo and in extracts was apparent (Fig. 3). Hence, breakdown of NTP during extraction was negligible. (Likewise, equivalent amounts of total sugar nucleotides were observed in vivo and in extracts [data not shown]. In contrast, the amount of NDP observed in vivo was always much lower than that found in extracts (Fig. 4). These results indicate that there is a sizeable pool of NDP in corn root tips that is not detected by in vivo NMR. The size of this NMR-invisible pool of NDP, given by the difference between the two data sets in Figure 4, showed little response to the metabolic state of the tissue, appearing quite similar in all samples examined. The increase in detectable NDP that was seen on extraction was similar in samples regardless of the amount of NTP (Figs. 3 and 4), a result consistent with the above conclusion that increased NDP was not due to NTP breakdown.

Proportions of Bases in Nucleotides are Insensitive to Metabolic State

While $^{31}$P-NMR can be used to observe nucleotides in vivo, it cannot distinguish between nucleotides having different

260 nm was multiplied by the ratio of $e_{260}$ (XMP/nucleotide), determined at the ionic strength and pH at which the compounds eluted from the column. This value was multiplied by the amount of XMP added to the sample (210 nmol/500 root tips).
bases. Hence, we examined root tip extracts by HPLC to determine how the relative amounts of each nucleotide changed with metabolic state. Under the diverse conditions used here, we found little change in the relative amounts of A, U, C, and G in NTPs, NDPs, and sugar nucleotides (Figs. 5–7). The largest change observed was in ATP, which increased from ~45% to ~60% of total NTP, as [NTP]/[NDP] increased.

**Evidence for Compartmentation of NDP from Analysis of the NDPK Reaction**

We examined the NDPK reaction in corn root tips by HPLC analysis of nucleotides in extracts. For nonadenylates, [NTP]/[NDP] displayed a linear dependence on [ATP]/[ADP] (Fig. 8). Each of the treatments giving lower [NTP]/[NDP] (exposure to KCN and/or low oxygen tension) act specifically at the level of oxidative phosphorylation. Hence, they must serve first to decrease [ATP]/[ADP]; decreases in [NTP]/[NDP] of nonadenylates must be a secondary response. The results indicate that the NDPK reaction is near equilibrium. Thus, the behavior of the data in Figure 8 is consistent with Le Châtelier's principle (2): when [ATP]/[ADP] is perturbed, nonadenylates respond in a way such that [NTP][ADP]/[ATP][NDP] is constant. The treatments employed in this study result in the rate of turnover of ATP varying over a ~10-fold range, decreasing as [NTP]/[NDP] decreases (22, 23). If the NDPK reaction was not near equilibrium *in vivo* then, as the rate of production of NDP and ATP decreased, [NTP][ADP]/[ATP][NDP] would tend to the equilibrium constant, since these fluxes would be responsible for any disequilibrium among reactants accessible to NDPK. Such behavior was not observed, suggesting near equilibrium of the NDPK reaction under all conditions.

However, one aspect of the results in Figure 8 is inconsistent with the idea of near-equilibrium of total cellular NTP and NDP via the NDPK reaction. The slopes of the lines for U and G are 0.63 and 0.42, respectively—significantly less than 1, the equilibrium constant for the NDPK reaction (14).
checked the value of this equilibrium constant by HPLC analysis of mixtures of NTPs and NDPs, incubated in the presence of 3 mm MgCl₂, 20 mm KPi, and 5 units/mL NDPK [Sigma] at pH 7.0 for 1 h. In three separate experiments using different initial nucleotide concentrations and ratios, we found mean equilibrium constants (±std) of 0.83 ± 0.01, 0.98 ± 0.05, and 0.93 ± 0.06 for U, G, and C, respectively. If the NDPK reaction is near-equilibrium, the non-ideal behavior in Figure 8 suggests the existence of pools of nucleotides sequestered away from NDPK, such that only a portion of the total cellular NDP could be equilibrated via the NDPK reaction. In the latter case, more UDP and, particularly, GDP would be inaccessible to NDPK, relative to other nucleotides, accounting for the fact that total cellular [GTP]/[GDP] < [UTP]/[UDP] < [CTP]/[CDP] ~ [ATP]/[ADP]. This is discussed below with respect to the results of our NMR experiments.

**DISCUSSION**

An important practical and long-recognized consequence of compartmentation of nucleotides in cells is that enzymes participating in metabolism can experience nucleotide concentrations and ratios very different from those observed in cell extracts (e.g. Ref. 10). The results presented above provide two lines of evidence for compartmentation of nucleotides in corn root tips. First, compartmentation with respect to NMR visibility. The amount of NDP seen in vivo is lower than in extracts (Fig. 4). Qualitatively similar results have been reported from NMR studies of human blood platelets (4, 28), skeletal muscle (7), and rat kidney (6), where it was concluded that NMR-invisible nucleotides present in vivo were immobilized; immobilized species give broad signals that are undetectable in high resolution experiments. Hence, existence of an immobilized pool of NDP in corn root tips is one possible explanation for the result in Figure 4. Such immobilized nucleotides contrast with nucleotides bound to certain enzymes, which retain significant mobility and therefore are visible by high resolution NMR (for review, see Ref. 24), as are "free" nucleotides. The chemical shifts of nucleotides may...
change only slightly on binding to enzymes, as in the case of ATP and arginine kinase (16), or significantly, for example the ~3 ppm shift in the β-GDP resonance on binding to elongation factor Tu from Escherichia coli (31). In vivo, NDP could be bound to many different proteins, each complex having a different chemical shift, so that the spectrum of such a mixed population of complexes could consist of a broad line. To date, the relative contributions of signals from “free” and enzyme-bound, but still freely mobile, nucleotides to in vivo 31P-NMR spectra have not been determined.

Second, we provide evidence for compartmentation of NDP with respect to accessibility to the enzyme NDPK, on the time-scale of nucleotide turnover (seconds). Results of HPLC analysis of extracts indicated near-equilibrium of the NDPK reaction (Fig. 8). However, for root tips in a given metabolic state, nucleotides containing U and, particularly, G were always less phosphorylated than the nucleotides containing A and C. We suggested that this behavior would occur if corn root tips contained a pool of NDP inaccessible to NDPK, this pool being enriched in GDP and UDP. The plausibility of this model can be tested, using the results of our 31P-NMR experiments. We postulate that the pool of NDPK, on the other hand, is rendered NMR-invisible by interaction with proteins, one can infer from Figures 4, 6, and 9 that (a) most of the NMR-invisible NDP binds to these proteins with dissociation constants much lower than the lowest concentration of NMR-visible NDP in vivo (~10^{-3} M); (b) the concentration of NDP-binding sites having dissociation constants ~10^{-4} M is ~0.15 μmol/500 root tips, the concentration of NMR-invisible NDP; and (c) the proportion of UDP and, particularly, GDP bound to these sites is higher than in the total NDP pool. It should be possible to test for the existence of such strong nucleotide-binding proteins in cell extracts (cf. for example, Ref. 8). The effect of these nucleotide-binding sites, and others that bind nucleotides less tightly, on the concentrations and ratios of free nucleotides in cells remains to be determined.

**LITERATURE CITED**