Partial $^{13}$C isotopic enrichment of nucleoside monophosphates: useful reporters for NMR structural studies

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ABSTRACT

Analysis of the $^{13}$C isotopic labeling patterns of nucleoside monophosphates (NMPs) extracted from Escherichia coli grown in a mixture of C-1 and C-2 glucose is presented. By comparing our results to previous observations on amino acids grown in similar media, we have been able to rationalize the labeling pattern based on the well-known biochemistry of nucleotide biosynthesis. Except for a few notable absences of label (C4 in purines and C3' in ribose) and one highly enriched site (C1' in ribose), most carbons are randomly enriched at a low level (an average of 13%). These sparsely labeled NMPs give less complex NMR spectra than their fully isotopically labeled analogs due to the elimination of most $^{13}$C-$^{13}$C scalar couplings. The spectral simplicity is particularly advantageous when working in ordered systems, as illustrated with guanosine diphosphate (GDP) bound to ADP ribosylation factor 1 (ARF1) aligned in a liquid crystalline medium. In this system, the absence of scalar couplings and additional long-range dipolar couplings significantly enhances signal to noise and resolution.

INTRODUCTION

The use of uniform, isotopic enrichment in biological molecules has been indispensable to the advancement of biomolecular NMR. Resonance assignment of $^{13}$C and $^{15}$N nuclei in proteins up to 40 kDa and nucleic acids up to 15 kDa is now routine in high resolution NMR (1–3). In solid-state NMR and in NMR of partially ordered systems, isotope labeling methods are also advantageous. In general, uniform isotopic labeling has facilitated assignment but has introduced some unique spectroscopic problems as well. For example, abundant scalar and dipolar couplings of adjacent $^{13}$C nuclei, actually degrade resolution and can lead to dilution of signal by transfer of magnetization through multiple pathways. Selective labeling reduces the probability of adjacent $^{13}$C groups, improving spectral resolution and simplifying resonance assignment.

Selective labeling is often achieved through exploitation of known metabolic pathways in organisms such as Escherichia coli. Such procedures have been primarily applied to protein systems (4,5); however, some selective labeling has also been done in nucleic acids (6,7), primarily for applications to dynamics. The high cost of selectively labeled late-stage intermediates has, nevertheless limited applicability. Labeling with random fractionally labeled early-stage substrates can be a more cost-effective alternative (8–10), but such randomly labeled metabolic substrates are not always available. Here we present a procedure for nucleotide labeling that relies on a combination of fully, but site-specifically, labeled early-stage substrates that may facilitate NMR structural application to RNAs and systems that use nucleotides as cofactors. Our own particular interest is in production of labeled guanosine diphosphate (GDP) for the study of GDP–GTP switch proteins. The anticipated structural data are from chemical shift anisotropy (CSA)-offsets and residual dipolar couplings. As an illustration we present some preliminary data on one such system.

$^{13}$C labeling of proteins using site-specifically labeled early-stage substrates has been documented previously in protein systems. For example, substitution of site-specifically labeled substrates, such as glucose (C1 and C2), glycerol (C1/C3 and C2) or pyruvate (C1 and C3), for uniformly labeled substrates leads to enhanced labeling at a subset of sites throughout expressed proteins (4,5,11–13). There can, of course, be some sacrifice in sensitivity by a reduction in percentage of labeling at certain sites, but as suggested above this is partially compensated by more efficient magnetization transfer and simpler spectra. Pulse sequences developed for magnetization transfer in uniformly enriched molecules typically require only minor modifications to take advantage of the spectral simplification seen in specifically labeled systems.
Nucleic acid structure determination has also benefited from isotopic labeling. Protocols to extract intact RNA from cells grown on uniformly isotopically labeled media and to isolate nucleoside monophosphates (NMPs) from the RNA have been described (6,14), but the use of specifically labeled precursors is less common (7,15). Isolating deoxyNMPs (dNMPs) is more difficult since cells contain 7–10 times more RNA than DNA (16), but this, too, has been described (17,18). Most protocols to extract NMPs from cell lysate call for the growth of *E.coli* in labeled minimal media to the late log phase for optimum ribosome production. Simple organic extractions easily separate proteins and lipids from the polymeric nucleic acids found in these structures (19).

Our initial motivation for the work described here included an attempt to take advantage of by-products from protein labeling efforts at the SECSG (20). Proteins expressed for the NMR core of the SECSG were grown in 98% 15N ammonium chloride and a mixture of 13C-1 and 13C-2 glucose instead of uniformly labeled glucose; this yielded carbon enrichment at the 16–20% level in targeted proteins. The primary justification for this method was to initially reduce costs in large scale expression, but additional spectroscopic benefits have also been described (21). As discussed below, isolation of dNMPs and NMPs from cell debris proved difficult. In cells that have already been harvested and extracted of proteins, the lysate is frequently treated with deoxyribonuclease (DNase I) to hydrolyze DNA and reduce the viscosity of the solution. In addition, most protein preparation protocols do not use RNase-free techniques to preserve the ribosome and other readily isolatable sources of RNA. The mononucleoside phosphates (dNMPs and NMPs) that result from DNase and RNase hydrolysis are more difficult to isolate and extract than intact nucleic acids. Hence, *E.coli* was grown to late log phase with the isolation of labeled nucleotides specifically in mind.

The use of 13C-1 and 13C-2 glucose for partial labeling remains an important aspect in the studies described here. The low percentage 13C enriched nucleotides isolated from *E.coli* grown with C-1 and C-2 labeled glucose should enjoy the same dilute spin advantages described for proteins. These nucleotides are particularly desirable over uniformly enriched nucleotides for measuring 13C CSA-offsets and RDCs in aligned systems. The alignment of biomolecules in the magnetic field has produced a wealth of information on their structures and orientations. RDCs induced by low levels of order have been measured in a variety of molecules and alignment media (22–25). Chemical shift offsets can provide orientation constraints in a manner quite analogous to those provided by RDCs. They are particularly advantageous in providing constraints on nucleotide bases where all RDCs are in the plane of the base and the out-of-plane contributions of CSA-offsets are highly complementary. CSA-offsets have been measured for a number of biological systems where ordering is weak and long-range dipolar couplings cause minimal degradation of resolution (26–31). In the case of a more strongly aligned sample, (in membrane-associated systems, e.g. expected changes in chemical shift between isotropic and aligned resonances of aromatic carbons could reach tens of p.p.m. (32–35). However, under such strong alignment, multiple through-space dipolar couplings present in uniformly labeled samples produce coupled spectra with many poorly resolved splittings. Even 13C–13C one-bond scalar couplings in uniformly labeled samples contribute an additional 40 Hz, and two- and three-bond couplings produce an additional 7–11 Hz (36). When through-space dipolar couplings are present, the splittings are even more numerous. Hence, improved resolution is expected for partially labeled nucleotides, particularly if labels are nearly randomly distributed.

One example of the potential use of partially labeled nucleotides is to measure 13C chemical shift offsets for nucleotide cofactors bound to membrane-associated GDP/GTP-binding GTPases or G proteins (37,38). RDCs have been used in combination with other NMR data to determine geometries of bound ligands in weakly aligned proteins (39–43), and it should be possible to use CSA-offset information in a similar way when systems are more strongly ordered. It is in these latter applications that partial labeling can be particularly advantageous.

RDCs from the protein itself have already been used to determine the molecular geometry of the membrane-associated GTase ADP ribosylation factor 1 (ARF1) (44) and its orientation in weakly aligned systems (45). When more strongly aligned through association with a membrane, the CSA-offsets from ARF’s isotopically labeled nucleotide cofactor GDP or GTP may be more accessible and may prove a more useful probe of protein orientation relative to the membrane surface. To pursue such studies, it is important to have methods for producing partially labeled nucleotides and to know the details of labeling patterns for such molecules. Here we offer a fundamental analysis of isotopic labels in nucleotides extracted from *E.coli* grown on a mixture of C-1 and C-2 labeled glucose. We also present spectra demonstrating the enhanced signal to noise and improved resolution of partially 13C-labeled GDP over uniformly labeled GDP when bound to ARF1, both in solution and in the presence of a model membrane. Although the extent of ordering in our particular system is less than in many membrane-anchored protein systems, it provides a stepping-stone to future studies of more strongly ordered systems with bound nucleotide cofactors as well as for large RNA systems.

**MATERIALS AND METHODS**

**Cell growth and nucleoside monophosphate (NMP) isolation**

All isotopes were purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA). M9 media for growth of cells was prepared by adding per liter: 6 g NaHPO₄, 3 g KH₂PO₄, 0.5 g NaCl and 1 g NH₄Cl to ddH₂O and the solution was autoclaved. In addition, filter-sterilized solutions of the following reagents were prepared for varying media additions required during expression (26–31). 2.5 g NaHPO₄, 0.5 g KH₂PO₄, 0.02 M CuCl₂·2H₂O and 0.01 M NiCl₂·6H₂O. A pET14 plasmid with no insert was transformed into *E.coli* BL21(DE3) Gold (Stratagene, La Jolla, CA) and plated onto M9 agar plates containing 100 μg/ml ampicillin. A single colony was used to inoculate 50 ml of M9 media containing 100 μg/ml ampicillin. A single colony was used to inoculate 50 ml of M9 media containing 100 μg/ml ampicillin. A single colony was used to inoculate 50 ml of M9 media containing 100 μg/ml ampicillin. A single colony was used to inoculate 50 ml of M9 media containing 100 μg/ml ampicillin.
20 ml (1:50 dilution) of the overnight culture. The flask was grown at 37°C while shaking at 250 r.p.m. until OD<sub>600</sub> = 0.7 (~3–4 h), moved to 22°C and the cells were harvested 16 h later. The cell pellets (~15 g) were immediately frozen at -70°C. Ribosomal RNA was extracted from thawed cells via French press, slow speed (SS-34) and high speed (Ti70) centrifugation and organic phase extraction according to Nkonowicz et al. (14). RNA was hydrolyzed to NMPs using P1 Nuclease (Roche), and the NMPs were eluted from a Vydacl 3021.10C ion exchange high-performance liquid chromatography (HPLC) column (Grace Vydacl, Hesperia, CA) with 50 mM ammonium formate, adjusted to pH 3.2 with formic acid (both from Aldrich, St Louis, MO). Peaks corresponding to CMP, UMP, AMP and GMP were collected separately, lyophilized, redissolved in water and lyophilized an additional two times to facilitate removal of volatile ammonium formate. Approximately 12 mg of each partially labeled NMP was recovered.

**GDP synthesis**

Since our target application is a GDP/GTP-binding switch protein (ARF1), GMP was converted to GDP, and for comparison purposes, this was done with both partially labeled and fully labeled starting materials. Fully labeled GDP was synthesized from >98% 13C, 15N GMP (Spectra Stable Isotopes, Columbia, MD), and partially labeled GDP was synthesized from the GMP described above, both using a modified protocol from Nkonowicz et al. (14). Guanylate Kinase (Sigma) was used to phosphorylate GMP and excess ATP (Sigma) was added as the phosphate donor. The reaction was maintained at 37°C for 44–48 h and then quenched by addition of 100 μl of 50 mM EDTA. The volume was reduced to half by lyophilization, and absolute ethanol was added to three times the volume with overnight storage at ~20°C to effect precipitation of nucleotides. The precipitated nucleotides were dried in air, dissolved in water and purified by HPLC using the Vydacl 3021.10C ion exchange column and a 35 ml step gradient of 50, 100 and 200 mM ammonium formate (pH 3.2). Fractions were lyophilized, redissolved in water and lyophilized again to facilitate removal of formate. The yield from this separation was 50–65%.

**Nucleotide exchange**

15N-labeled ARF1 was prepared according to methods described previously (44). 13C and 15N GDP was exchanged into ARF1 by an EDTA-mediated exchange. A 25-fold excess of EDTA over ARF1 was added to a solution of 0.5 mM ARF1 to remove magnesium and facilitate turnover of native nucleotide. This was followed by addition of a 10-fold excess of 13C and 15N GDP in buffer containing 20 mM Tris–HCl (pH 7.6), 100 mM NaCl, 5 mM MgCl₂ and 2 mM NaN₃. The exchange took place for 48 h at 4°C. EDTA was removed from the solution by several serial dilutions with the above buffer minus GDP until the final estimated concentration of EDTA was <30 mM.

**Alignment medium preparation**

C₁₂E₅₆/hexanol has previously been identified as a suitable alignment medium for both ARF1 (45) and a lipid that interacts with ARF1 at a membrane surface, PIP₂ (46). This medium was prepared as follows: Pentaethylene glycol monododecyl ether C₁₂E₅₆ (Sigma, St Louis, MO) was combined with hexanol in the ratio 0.88:1 to make a solution of 4.6% (w/w) C₁₂E₅₆ (47–49). Pure PIP₂ [phosphatidylinositol 4,5-bisphosphate, Avanti Polar Lipids, (Alabaster, AL)], was dried under a stream of nitrogen and added directly to the C₁₂E₅₆ bilayers at a relatively low molar ratio of PIP₂ to C₁₂E₅₆ (1:80) so that bilayer order is not disrupted. ARF1 was added to the medium to produce a final solution of ~0.7–1.0 mM protein at a 2:1 PIP₂ to ARF1 ratio.

**NMP NMR analysis**

NMP fractions were analyzed by 1H, 13C and 31P NMR on a 500 MHz Varian (Palo Alto, CA) Inova spectrometer using a triple resonance Varian probe for 1H, a 5 mm Nalorac 13C/15N broadband observe probe (operating at 125.6 MHz) for 13C, and a 5 mm Varian broadband observe probe (operating at 202.6 MHz) for 31P spectra. 13C spectra were collected with a 45° tip angle, rapid recycling (0.5 s recycle delay), and Waltz-16 1H decoupling for NOE enhancement. All spectra were collected at 25°C. These spectra were used to determine chemical shifts (and 13C–H scalar couplings by turning off the proton decoupling during acquisition). Relative peak intensities were determined by integrating peaks observed with decoupling during acquisition only and a long recycle delay (5 s) to allow sufficient magnetization recovery. 31P observe and 1D 1H–31P HMOC spectra were collected to confirm ribose phosphorylation (data not shown). The pH of each sample was recorded, and free phosphate was added to 0.85–1.65 mM to each HPLC fraction as a reference signal for determination of 31P chemical shift and nucleotide concentration. 13C chemical shifts were referenced indirectly by referring to added DSS and using appropriate ratios for properly referenced 13C spectra (50,51).

**GDP-Bound ARF1 NMR analysis**

13C direct observe data on an ARF1 complex containing labeled GDP were collected at 25°C on an 800 MHz Varian Inova spectrometer using the normal 13C decoupling input on a triple resonance Varian Chilli Probe in a 4 mM D₂O susceptibility-matched Shigemi (Alison Park, PA) tube. 13C spectra were collected with a 33–45° tip angle and Waltz-16 1H decoupling at a B₁ field of 12.000 Hz during acquisition. For 98% 13C-GDP isotropic samples, 30,000–40,000 scans were acquired, and for aligned samples 83,000 scans were acquired, both with a 1.6 s recycle time. For 13C 13C-GDP isotropic samples, 300,000 scans were acquired, and for aligned samples 630,000 scans were acquired, both with rapid recycling (0.3 s recycle time). 15N–1H heteronuclear single quantum coherence spectra (HMQC) were also collected before and after each 13C experiment (data not shown) to confirm protein integrity. Liquid crystal alignment was also confirmed by monitoring the ²H NMR quadrupolar splitting of water deuterons (18–21 Hz). Protein alignment was independently determined by measuring 14N–1H RDCs on the lipid doped C₁₂E₅₆ model membrane sample (data not shown). 13C spectra were processed using NMRPipe (52), applying backwards linear prediction and an exponential apodization function with a 30 Hz line broadening constant. The automated lineshape fitting routine within NMRPipe (nlmLS) was used in...
order to accurately extract resonance positions. $^{13}$C and $^{15}$N chemical shifts in the protein samples were referenced indirectly by referencing to added DSS and using appropriate ratios for properly referenced $^{13}$C or $^{15}$N spectra, respectively.

RESULTS

In Figure 1 the $^{13}$C NMR spectrum of both the base and ribose regions for UMP is shown as a representative of other nucleotide spectra. Chemical shift assignments are based on previously reported data (36). Note, that the C1′ carbon shows the highest level of carbon labeling. Peak intensities vary due to the differing levels of enrichment, as dictated by their origin in biosynthetic pathways. Most other ribose and base carbons show moderate levels of labeling as evidenced by $^{13}$C spectra; the lack of $^{13}$C–$^{13}$C couplings (except at the bases of peaks, e.g. to C5 shown in Figure 1 inset) also shows low probabilities of simultaneous labeling at adjacent sites.

In Figure 2 expansions of the base region in $^{13}$CN M R spectra of each of the NMPs are shown. C5 and C6 of pyrimidines (Figure 2a for CMP, Figure 2b for UMP) appear to get labeled at approximately the same percentage, and C2 and C4 show significantly lower intensities. These differing intensities are due, in part, to the nuclear overhauser enhancement (NOE) for protonated carbons, C5 and C6. A small amount of AMP was present in the UMP spectrum, as noted by asterisks. In purine (Figure 2c for AMP, Figure 2d for GMP) spectra, we observed $^{13}$C labeling of C2, C5, C6 and C8 and almost no $^{13}$C labeling of C4 (Figure 2c and d). C2 and C8 show similar labeling levels in both AMP and GMP. C5 and C6 exhibit lower, but approximately equal levels of isotopic labels. The AMP fraction was contaminated with about 10% UMP, represented in Figure 2c by asterisks. Several one-bond $^{13}$C–$^{15}$N scalar couplings are observed in $^{13}$C spectra, e.g. the barely observable splittings seen at the top of the peaks for C4 and C6 of UMP and CMP (Figure 2a and b).

In Table 1 we summarize the chemical shifts for the four NMPs as well as estimates of isotopic enrichment. We estimate the level of carbon enrichment based on $^{13}$C resonance intensities collected with long recycle delays (data not shown) and on $^{13}$C satellites in $^1$H spectra (Figure 3). The H6 proton signal from CMP (Figure 3a), e.g. shows $^{13}$C satellites that represent 29% of the total incorporation. H1′ is the only ribose sugar proton resonance with appreciable $^{13}$C satellites. Satellites for H5 and H1′ resonances (Figure 3b) of CMP show about 31% $^{13}$C incorporation for H5 and 45% for H1′.

In Figure 4a and b we present $^{13}$C spectra from the aromatic region of $^{13}$C-labeled GDP-bound to ARF1 when aligned (Figure 4a) and in isotropic buffer solution (Figure 4b), respectively. The aligned sample is oriented in a 4.6% (w/w) C12E5 bilayered liquid crystal doped with PIP3, a signaling lipid that is suggested to interact with ARF1 (53–55). Aromatic base carbon frequencies are labeled for easily observed resonances; C4 and C6 are not observed due to lower levels of isotopic incorporation. Unlabeled regions of high spectral intensity represent natural abundance $^{13}$C protein signals from aromatic (~130 p.p.m.) resonances; these are marked with double asterisks. Some free GDP (denoted by a single asterisk) is also observed in isotropic solution, and resonances from this free GDP are readily identified based on distinct chemical shifts between free and bound forms. Chemical shift offsets between isotropic and aligned spectra, though small, are
observed for the aromatic carbons, with C2 and C5 displaying differential shifts with C2 moving downfield (54 Hz). These differences are likely to arise from orientational dependences of CSAs, and they can be structurally useful.

The resolution and quality of some signals, e.g. C2, is also significantly better than what can be obtained under comparable conditions with a 98% carbon-labeled sample. Figure 5a and b show expansions of the C2 region of the spectrum for similarly aligned samples using a 13% carbon-labeled sample (Figure 5a) and a 98% carbon-labeled sample (Figure 5b). The resonance is considerably broader for the 98% sample and the signal to noise ratio appears less despite the fact that the additional scans for the 13% sample (a factor of 8) are not nearly enough to compensate for the decrease in 13C content by a factor of 7.5. Even if repetition times were the same in both acquisitions (it was shorter for the 13% sample), this factor would need to be squared to fully compensate for decreased label content.

**DISCUSSION**

Improved resolution in spectra of partially labeled NMPs

There is little doubt that reduction of percent labeling, when done approximately randomly, results in an improvement in signal quality despite somewhat lower overall sensitivity. We suggest that the absence of significant 13C–13C splittings from both scalar and long-range dipolar couplings is the origin of this improvement. One would expect broadening of resonances and loss of signal due to unresolved splittings of resonances when enrichment is high or if labeled pairs were

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**Table 1.** 13C chemical shifts (p.p.m.) of labeled NMPs and site-specific percentage of isotopic incorporation

| 13C | CMP | UMP | AMP | GMP |
|-----|-----|-----|-----|-----|
|     | Chemical shift (p.p.m.) | Relative % label<sup>a</sup> | Absolute % label<sup>b</sup> | Chemical shift (p.p.m.) | Relative % label<sup>a</sup> | Absolute % label<sup>b</sup> | Chemical shift (p.p.m.) | Relative % label<sup>a</sup> | Absolute % label<sup>b</sup> | Chemical shift (p.p.m.) | Relative % label<sup>a</sup> | Absolute % label<sup>b</sup> |
| C2  | 159.8 | 32 | 14 | 154.5 | 39 | 16 | 155.1 | 73 | 38 | 156.8 | 66 | 22 |
| C4  | 168.5 | 46 | 21 | 168.9 | 41 | 16 | 151.5 | 7 | 4 | 14 | – | <2 |
| C5  | 99.3 | 68 | 31 | 105.2 | 72 | 29 | 121.2 | 40 | 21 | 118.9 | 37 | 12 |
| C6  | 144.5 | 64 | 29 | 144.5 | 61 | 24 | 157.9 | 31 | 16 | 161.8 | 28 | 9 |
| C8  | – | – | – | – | – | – | 142.6 | 70 | 36 | 140.3 | 57 | 19 |
| C1' | 92.1 | 100 | 45 | 91.3 | 100 | 40 | 89.9 | 100 | 52 | 89.6 | 100 | 33 |
| C2' | 77.0 | 26 | 12 | 76.5 | 30 | 12 | 77.0 | 26 | 14 | 76.6 | 26 | 9 |
| C3' | 72.3 | 8 | 4 | 72.4 | 11 | 4 | 73.1 | 5 | 3 | 73.2 | 7 | 2 |
| C4' | 85.8 | 29 | 13 | 86.3 | 35 | 14 | 86.7 | 24 | 12 | 87.7 | 35 | 12 |
| C5' | 66.3 | 63 | 28 | 66.4 | 58 | 23 | 66.8 | 52 | 27 | 67.2 | 42 | 14 |

<sup>a</sup>Percentage labeling determined relative to C1 peak intensity by integrating 13C peak intensities in fully relaxed 13C spectra.

<sup>b</sup>Percentage determined by integrating 13C satellites to H1 in 1H spectra and using relative 13C percentage labels.
incorporated as intact units. We do not see scalar couplings at 13% enrichment under high resolution, non-protein-bound, conditions (Figures 1 and 2) and we would expect fewer dipolar couplings under aligned conditions as well. Resonance intensity, even if reduced by partial labeling, would be largely concentrated into a single resonance resulting in an improvement in resonance quality.

Biosynthetic pathways lead to desirable labeling:
Pentose Phosphate Pathway (PPP) yields labeled ribose

Near random labeling, or labeling only of selected sites, is essential to the gains from the absence of scalar or dipolar coupling described above. The labeling patterns observed for the systems studied here can be understood based on well-known biosynthetic pathways for ribose and the nucleotide bases. The use of $^{13}$C NMR has long been important in the elucidation of metabolic pathways (56–58) as well as in the introduction of $^{13}$C isotopic labels into proteins of interest. Less discussion exists for isotopic labeling of nucleotides, and none exists specifically for the use of C-1 and C-2 glucose mixture in E.coli growth. Here we attempt to rationalize the isotopic labeling pattern of nucleic acids grown with $^{13}$C labeling from C-1 and C-2 glucose.

Unlike most amino acids of proteins, where $^{13}$C labels from C-1 and C-2 glucose mixtures are nearly uniformly distributed across alpha, carbonyl and other carbons, ribose carbons are highly enriched at the C-1 position. Ribose-5-phosphate (R5P), the precursor to ribose in all nucleotides is produced directly from glucose and glucose-6-phosphate (G6P) in the PPP by the elimination of C-1 from glucose (Figure 6). Depending on the cell’s metabolic needs, 6GP can either undergo isomerization to fructose 6-phosphate (F6P) by phosphoglucone isomerase or oxidation and hydrolysis to 6-phosphogluconate (6PG) by

Figure 3. $^1$H NMR of CMP’s (a) aromatic region (H6) and (b) anomeric region (H5 and H1’). $^{13}$C satellites are observed for all three $^1$H peaks and are represented by a single asterisk for H5 and H6 and a double asterisk for H1’. Assignments were based on published spectra (36).
6GP dehydrogenase and 6-phosphoglucono lactonase, respectively. The subsequent oxidative decarboxylation of 6PG by phosphogluconate dehydrogenase to ribulose-5-phosphate (Ru5P) is the final step in the oxidative portion of the pentose phosphate pathway. Isomerization of Ru5P to ribose-5-phosphate (R5P) by Ru5P isomerase is controlled by the cell’s metabolic needs. R5P is then utilized in nucleotide biosynthesis (59). Hence, the enriched carbon of the C-2 glucose used in our minimal media for E.coli growth has a direct route to the C1 of ribose, and nucleotides thus become highly enriched at C1₀, as shown in Table 1.

Some labeling of ribose carbons C2₀, C4₀, and C5₀ is also observed (Figure 2), but C3₀ shows almost no ¹³C enrichment (Table 1). The labeling pattern can be rationalized as follows. The recycling of cellular components with near random levels of isotopic incorporation results in low labeling levels for most ribose carbons. Specifically, glycolysis and gluconeogenesis scramble the C-1 and C-2 labels such that C1₀, C2₀, C5₀, and C6₀ of glucose all become labeled. In addition, the PPP regenerates glucose with C1₀ and C3₀ labels (60–62). However, C4₀ does not become labeled through any of the above pathways. The final labeling scheme results in isotopic labels everywhere except C4₀ of glucose. Due to the decarboxylation discussed above, C4₀ of glucose becomes C3₀ of ribose, and little labeling of ribose C3₀ is observed.

**Purine base biosynthesis**

Labeling patterns in nucleotide base biosynthesis can be understood by considering isotopic labeling of their precursors. In the case of purines, labeling can be rationalized by...
considering the origin from constitutive amino acids, formate and bicarbonate (Figure 7a). C2 and C8 are derived from a formyl group transferred by N10-formyl-tetrahydrofurate. C6 is derived from bicarbonate. These one carbon sources are labeled by metabolic degradation of glucose. The single carbon pool is diluted by 12C carbons, hence labeling of these labeled by metabolic degradation of glucose. The single carbon source is derived from bicarbonate. These one carbon sources are labeled by metabolic degradation of glucose. The single carbon pool is diluted by 12C carbons, hence labeling of these labeled by metabolic degradation of glucose. The single carbon source is derived from bicarbonate. These one carbon sources are labeled by metabolic degradation of glucose. The single carbon pool is diluted by 12C carbons, hence labeling of these labeled by metabolic degradation of glucose. The single carbon source is derived from bicarbonate. These one carbon sources are labeled by metabolic degradation of glucose. The single carbon pool is diluted by 12C carbons, hence labeling of these labeled by metabolic degradation of glucose. The single carbon source is derived from bicarbonate. These one carbon sources are labeled by metabolic degradation of glucose. The single carbon pool is diluted by 12C carbons, hence labeling of these labeled by metabolic degradation of glucose. The single carbon source is derived from bicarbonate. These one carbon sources are labeled by metabolic degradation of glucose. The single carbon pool is diluted by 12C carbons, hence labeling of these labeled by metabolic degradation of glucose. The single carbon source is derived from bicarbonate. These one carbon sources are labeled by metabolic degradation of glucose. The single carbon pool is diluted by 12C carbons, hence labeling of these labeled by metabolic degradation of glucose.
through myristoyl chain—C12E2 bilayer interactions and display much larger shift offsets. This would give a picture of membrane–protein interaction geometry through the nucleotide cofactor reporter. We expect the partially labeled material and the approach to its production described here to be useful in such studies.

CONCLUSIONS

We have presented the 13C isotopic labeling patterns of NMPs extracted from E.coli grown on a less commonly used mixture of C-1 and C-2 glucose. By comparing our results to previously observed data on amino acids grown in similar media, we have been able to rationalize the labeling pattern. Despite a few notable absences (C4 in purines and C3’ in ribose) and one more highly labeled site (C1’ in ribose), most carbons are randomly labeled at a low level. With the low level of labeling, scalar couplings and additional splitting from long-range dipolar couplings are minimized, affording greater spectral quality than seen in fully labeled materials. This should allow high resolution measurement of structurally useful parameters such as chemical shift anisotropy-offsets in weakly aligned liquid crystals. Application to studies of proteins with nucleotide cofactors, particularly in their membrane-associated states where direct observation of 13C is preferred to observation of more strongly relaxing proton signals, should soon be possible.

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