Ca^{2+} and Proton Transport in Chromaffin Granule Membranes: A Proton NMR Study†

Poksyn S. Yoon and Robert R. Sharp*
Department of Chemistry, The University of Michigan, Ann Arbor, Michigan 48109
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ABSTRACT: High-resolution proton NMR spectroscopy has been used to monitor the internal pH of chromaffin granule ghosts during Ca^{2+} influx through the membrane. For this purpose, ghosts were prepared by lysing and resealing chromaffin granules in a medium containing the disodium–ethylenediaminetetraacetic acid complex (Na_2·EDTA). Uncomplexed EDTA and Ca·EDTA give rise to distinct sets of methylene peaks in the proton NMR spectrum. Free EDTA titrates with a pK near 6.6 in deuterated media; the chemical shifts that accompany titration have been used to monitor intravesicular pH changes which occur inside chromaffin granule ghosts as a result of (1) ATPase activity and (2) deprotonation of EDTA during Ca^{2+} influx and complex formation. ATPase activity results in an NMR-detectable proton gradient which is dissipated by nigericin. Experiments monitoring Ca^{2+} uptake showed that protons which are liberated inside ghosts as a result of Ca·EDTA complex formation are not extruded from the ghosts via a process coupled to Ca^{2+} entry. This suggests that the Ca^{2+} transport system of the chromaffin granule membrane occurs without concurrent proton antiport and is not directly coupled energetically to the transmembrane pH gradient.

Chromaffin granules, which are the specialized storage organelles of catecholamines in the adrenal medulla, contain high concentrations of Ca^{2+} [20 mM (Borowitz et al., 1965; Phillips et al., 1977)] and Mg^{2+} [7 mM (Phillips et al., 1977)]. The significance of these cations is not completely understood. It has frequently been proposed that Ca^{2+} plays a structural role in catecholamine–nucleotide aggregates which provide osmotic stabilization of the chromaffin granule interior (Berneis et al., 1969, 1970; Morris et al., 1977; Daniels et al., 1978; Granot & Rosenheck, 1978), although direct osmotic measurements do not provide strong support for such a role (Sen & Sharp, 1982; Kopell & Westhead, 1982). More recently, a Ca^{2+} carrier has been demonstrated in the membranes of intact chromaffin granules (Kostron et al., 1977; Krieger-Brauer & Gratzl, 1981, 1982; Niedermaier & Burger, 1981; Hausler et al., 1981) and in chromaffin granule ghosts (Phillips, 1981; Krieger-Brauer & Gratzl, 1983). The presence of this carrier, which is apparently linked to endogenous Na^+ gradients, suggests that chromaffin granules may play a central role in Ca^{2+} homeostasis.

The driving force for Ca^{2+} transport is not entirely clear. Calcium uptake is apparently linked to an opposing Na^+ gradient both in intact chromaffin granules (Krieger-Brauer & Gratzl, 1981) and in ghosts (Phillips, 1981), although the dependence of cation uptake upon the protonmotive force is controversial (Kostron et al., 1977; Phillips, 1981; Grafenstein & Neumann, 1983; Hausler et al., 1981; Niedermeyer & Burger, 1981). The purpose of the experiments reported here was the development of an NMR method suitable for observing Ca^{2+} uptake by chromaffin granule ghosts with a simultaneous measurement of the lumenal pH. For this purpose, we have prepared chromaffin granule ghosts by lysing and resealing the membranes in media containing ethylenediaminetetraacetic acid (EDTA).[1] The chemical exchange reaction Ca^{2+} + EDTA = Ca^{2+}·EDTA is slow on the NMR time scale, and the proton NMR resonances of the two chemically distinguishable methylene groups of Ca^{2+}·EDTA are distinct from those of uncomplexed EDTA. Free EDTA titrates in the region of pH 6.6, and the proton resonances of this species undergo titration chemical shifts which provide a convenient probe of the lumenal pH. The resonances due to external and internal EDTA are easily distinguished in the presence of excess external Ca^{2+} since all of the external EDTA exists as the Ca·EDTA complex, while the internal EDTA is initially free, and subsequently is complexed only as a result of Ca^{2+} entry into the lumen. Thus, the EDTA peaks provide a simultaneous measurement of the Ca^{2+} content and the pH of the ghost lumen.

The results show that protons which are liberated inside ghosts as a result of Ca·EDTA complex formation are not extruded from the ghosts via a process coupled to Ca^{2+} entry. This suggests that the Ca^{2+} transport system of the chromaffin granule membrane is not directly coupled to the protonmotive force.

Materials and Methods

Adenosine 5′-triphosphate (ATP), BES, EDTA, and bovine serum albumin were obtained from Sigma Chemical Co., DSS was from Aldrich Chemical Co., and Me_2SO-d_6 was from Merck Sharp & Dohme.

Preparation of Bovine Chromaffin Granules. A crude chromaffin granule pellet was prepared according to the procedure described previously (Smith & Winkler, 1967) except that the isolation medium contained 0.3 M sucrose and 20 mM BES, pH 7.2. Chromaffin granules were further purified by sedimentation through a ficoll–sucrose step gradient (Trifaro & Dworkind, 1970) in the following manner. The crude pellets were resuspended, combined, and sedimented at 20000g for 30 min at 8 °C. For all preparations containing D_2O, the temperature was maintained above 5 °C to prevent freezing of the solvent. The upper layer of the ficoll–sucrose

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[1] Abbreviations: pH*, measured pH in D_2O solvent; BES, *N*,*N*-bis-(2-hydroxyethyl)-2-aminoethanesulfonic acid; KP_i, potassium phosphate buffer; DSS, 4,4-dimethyl-4-silapentane-1-sulfonic acid; FCCP, carbonyl cyanide *m*-fluorophenylhydrazone; Me_2SO-d_6, perdeuteriodimethyl sulfoxide; EDTA, ethylenediaminetetraacetic acid; BSA, bovine serum albumin.

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gradient, which contained the mitochondria and lysosomes, was aspirated off, and the chromaffin granule contained pellet was washed twice with fresh buffer. The procedure yielded preparations which routinely had catecholamine to protein ratios greater than 0.40.

Preparation of Chromaffin Granule Ghosts. Fresh bovine chromaffin granules, prepared as described above, were lysed within 48 h after slaughter to obtain chromaffin granule ghosts according to a modification of the procedure of Njus & Radda (1979). Granules were lysed and resealed by incubation of the resuspended pellet in a 10-fold excess by volume of 50 mM KP_i, pH 7.2, containing 10% (v/v) glycerol and 50 mM Na_2EDTA or K_2EDTA as indicated. The suspension was allowed to equilibrate for 30 min at 0 °C with gentle stirring and was then dialyzed against 2 L of 50 mM KP_i, pH 7.2, and 50 mM K_2SO_4 for 2 h at 4 °C to remove glycerol. The ghosts were then sedimented by centrifugation at 30000*g* for 30 min and gently resuspended in 200 mM KP_i, pH* 7.2, and 80 mM K_2SO_4 in D_2O. These concentrations of buffer and impermeant anion were found to best stabilize the intact ghosts in D_2O solvent. Following resuspension, ghosts were again sedimented at 30000*g* for 30 min, and this wash procedure was repeated twice to ensure thorough deuteration of the solvent. The final resuspension buffer for ghost preparations contained 80 mM K_2SO_4 and 0.2% (w/v) bovine serum albumin, pH* 7.2. BSA was first dialyzed extensively against deionized water and lyophilized prior to use to remove an NMR-detectable impurity. Before final resuspension, ghosts were stable up to 2 days when stored at 0 °C. Protein concentrations of ghost preparations were determined by the method of Bradford (1976) or Lowry et al. (1951) and adjusted to approximately 9.0–10.0 mg of protein/mL prior to use.

High-Resolution NMR Measurements. ^1H NMR spectra (200 scans, 8192 data points) were obtained at 360.13 MHz by using a Bruker WM-360 NMR spectrometer under conditions of homonuclear decoupling for solvent suppression. In the case of Ca^{2+} uptake only (Figure 3), a spin-echo sequence, $(90°-\tau-180°-\tau-\text{accumulate})_n$, τ = 20 ms, was used to provide better suppression of resonances due to high molecular weight species. Chemical shifts are reported relative to internal DSS.

The stoichiometry of H^+ release during Ca·EDTA chelation was determined by pH titration of a standard solution containing 20 mM imidazole plus 2 mM EDTA, pH 7.0, and with a stock solution of 100 mM $Ca(NO_3)_2$ in 20 mM imidazole buffer, pH 7.0. This titration indicated that a single mole of protons is liberated per mole of Ca·EDTA formed.

ATP-dependent acidification of the ghost interior was monitored by using K_2EDTA-loaded ghost preparations (0.5 mL) containing 4.5–6.0 mg of protein. This suspension was treated with $Ca(NO_3)_2$ (10 mM final concentration) to chelate external EDTA, and the spectrum was recorded at 25 °C. This procedure provided a routine diagnostic test for the presence of intact, EDTA-containing chromaffin granule ghosts. The ghost sample was then incubated with 5 mM Na_2ATP, 5 mM $MgSO_4$, and 25 mM KCl, thereby initiating ATPase-dependent H^+ translocation, and NMR spectra were obtained at intervals of several minutes. After 20 min at 25 °C, nigericin in deuterated Me_2SO was added to a concentration of 4 μg/mg of protein, and a final spectrum was recorded. The lumenal EDTA concentration was estimated to be approximately 40 mM on the basis of a value of 4 μL/(mg of protein) for the volume of the lumen.

Ca^{2+} uptake was monitored by using Na_2EDTA-loaded ghost preparations (0.5 mL) containing 5 mg of protein at 37 °C. After a base-line spectrum was recorded, the suspension was made 10 mM in $Ca(NO_3)_2$, and spectra were recorded at 5-min intervals.

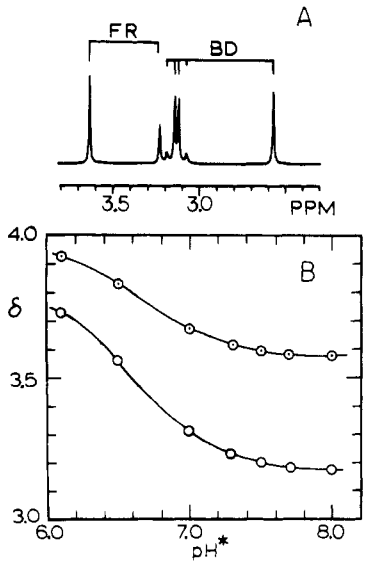

FIGURE 1: (A) ^1H NMR spectrum of 50 mM Na_2EDTA plus 30 mM $Ca(NO_3)_2$, pH* 7.13, in D_2O. Free and bound EDTA peaks are indicated. The aceto-CH_2 protons are to high field of the $(N-CH_2)_2$ protons. (B) ^1H NMR pH titration of EDTA. The pH* of a standard solution of 50 mM Na_2EDTA plus 25 mM K_2HPO_4 in D_2O was adjusted to the indicated values by addition of 1 M DCl (25 °C). Chemical shifts are referenced to internal DSS.

RESULTS

A solution containing a mixture of free EDTA and the Ca·EDTA complex shows separate pairs of peaks for the two species (Figure 1A). Unbound EDTA is characterized by peaks at 3.22 and 3.63 ppm, while Ca·EDTA gives rise to a downfield quartet at 2.56 and 3.11 ppm. Free EDTA titrates with a pK_a near 6.6 in deuterated aqueous media (Sillen & Martell, 1971), and in this range, the peaks of the two chemically distinct methylene protons shift to high field. This titration shift provides a convenient probe of lumenal pH and was calibrated by using standard solutions as shown in Figure 1B. In further titration experiments (data not shown), it was found that a single proton is lost on formation of the Ca·EDTA complex.

To demonstrate that the lumenal pH of chromaffin granule ghosts is measured by the chemical shifts of the resonances of EDTA which had been resealed inside the ghosts, control experiments were conducted monitoring the response of these resonances to Mg·ATP. In the presence of permeant anions, Mg·ATP acidifies the chromaffin granule lumen due to the action of the proton-translocating ATPase (Njus & Radda, 1977). The effect of Mg·ATP on the NMR spectrum of chromaffin granule ghosts which had been sealed in the presence of 50 mM K_2EDTA is shown in Figure 2. Free and bound EDTA peaks are indicated in the figure. In these experiments, sufficient Ca^{2+} was added in order to complex all of the external EDTA. Thus, the bound peaks arise only from external EDTA, and the free peaks reflect only internal EDTA, which is sequestered from added Ca^{2+}. Following the addition of 5 mM ATP, 5 mM $MgCl_2$, and 25 mM KCl, the free peaks were observed to broaden and shift upfield, indicating an acidification of the lumen. The internal EDTA peaks are substantially broadened as well as shifted, reflecting pH heterogeneity in the population of ghosts. Concurrently, a peak at 4.02 ppm due to ADP (the C-5′ methylene protons) grew as the hydrolysis of ATP proceeded. Two other peaks which

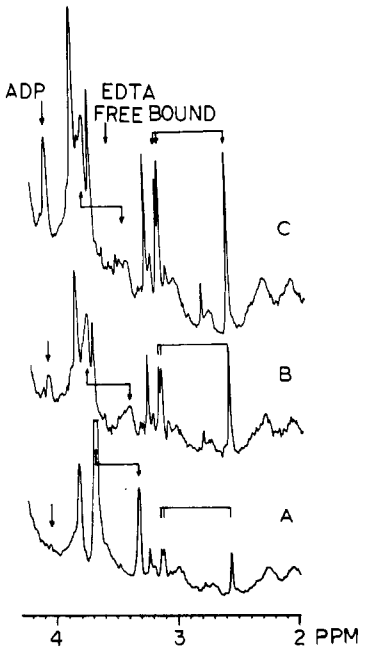

FIGURE 2: ^1H NMR spectra of EDTA-loaded chromaffin granule ghosts following addition of Mg·ATP: EDTA-loaded ghosts (external pH* 6.9, 25 °C) after the addition of (A) 10 mM $Ca(NO_3)_2$; (B) same as (A) plus 5 mM ATP, 5 mM $MgSO_4$, and 25 mM KCl at 7-min incubation; (C) same as (B) at 12-min incubation. Relative gains of spectra in (A), (B), and (C) are 0.5, 1.0, and 2.0, respectively.

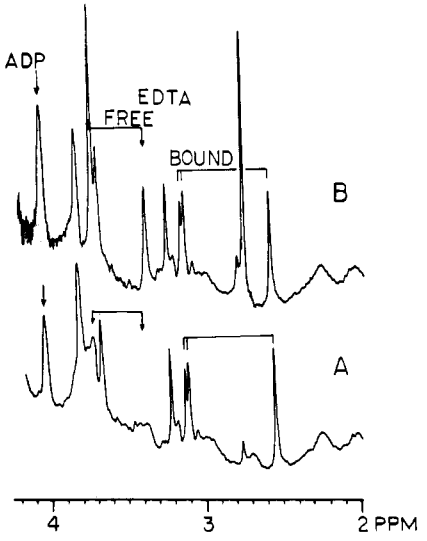

FIGURE 3: Proton NMR spectra of EDTA-loaded ghosts after additions indicated in Figure 2A–C: (A) after 20-min incubation; (B) plus nigericin (4 μg/mg of protein) in Me_2SO-d_6. The peak at 2.70 ppm is residual protonated Me_2SO.

partially obscure the low-field EDTA peak in the region 3.7–3.8 ppm arise from glycerol used in the ghost preparation. These peaks, as well as the peaks due to bound EDTA, were essentially invariant during the incubation.

ATP hydrolysis was essentially complete after 20 min, and the observed pH change was about 0.12 unit (Figure 3A). After 25 min, nigericin was added to collapse the ΔpH (Figure 3B). The free peaks shifted to higher field, indicating alkalinization of the lumen. These peaks also narrowed dramatically after nigericin addition due to pH equilibration among the environments containing free (internal) EDTA. A comparison of chemical shifts in spectra recorded prior to ATP addition (Figure 2A) and after addition of nigericin (Figure 3B) shows that net acidification of the medium has occurred. This is expected due to proton liberation during ATP hydrolysis. These experiments indicate that chemical shifts of the free EDTA peaks are responding to pH changes in the ghost lumen and that the nonequilibrium pH gradient produced in the ghosts by ATP hydrolysis is maintained from a period of several minutes.

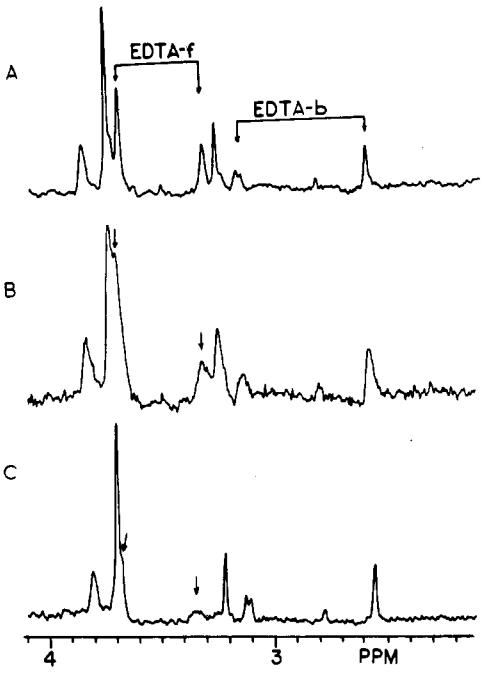

FIGURE 4: ^1H NMR spin-echo spectra of calcium uptake into EDTA-loaded ghosts (external pH* 7.0). (A) Base-line spectrum of ghosts (400-μL sample containing 10 mg of protein/mL, 37 °C). At time zero, $Ca(NO_3)_2$–D_2O was added to a final concentration of 10 mM. (B) Spectrum after 3-min incubation; (C) after 13-min incubation.

Table I: Change in Internal pH (ΔpH) and Change in Chemical Shift (Δδ) of the Upfield Peak of Free EDTA Inside Chromaffin Granule Ghosts following the Indicated 12-min Incubations[a]

preparation	additions	Δδ	ΔpH
K_2·EDTA	Ca_2NO_3	+0.09 ± 0.02	−0.14 ± 0.04
K_2·EDTA	Ca_2NO_3, Mg·ATP, KCl	+0.08 ± 0.02	−0.12 ± 0.03
Na_2·EDTA	Ca_2NO_3	0.00 ± 0.02	0.00 ± 0.03

[a] Ghost preparations were lysed and resealed in 25 mM KP_i plus 50 mM Na_2EDTA or K_2EDTA. Concentrations were 5 mM $MgSO_4$, 5 mM Na_2ATP, 30 mM KCl, and 10 mM $Ca(NO_3)_2$.

Subsequently, a suspension of ghosts that had been lysed and resealed in the presence of 50 mM Na_2·EDTA was incubated in the presence of 10 mM $Ca(NO_3)_2$ at 37 °C (Figure 4). Under similar conditions, Phillips (1981) has reported rapid Ca^{2+} uptake by ghosts. The free EDTA peaks were found to broaden and shift to higher field over a period of minutes. The integrated intensities of the free peaks decreased, and those of the Ca·EDTA complex increased. These changes reflect the entry of Ca^{2+} into the ghosts and a simultaneous acidification of the lumen. The chemical shift corresponds to a pH drop from 7.28 to 7.05 over a period of 13 min. (In these spectra, peak integrals are only qualitative measures of concentration because of T_2 relaxation during the spin-echo sequence.)

Similar Ca^{2+} incubations (Figure 5, Table I) were conducted at 25 °C in the absence of a resting Na^+ gradient using ghosts lysed and resealed in the presence of 50 mM K_2EDTA and resuspended in buffer contained 80 mM K_2SO_4 and 0.2% BSA, pH 7.2. Previous experiments have shown that Ca^{2+} movements are dependent on the presence of an opposing Na^+ gradient in both intact chromaffin granules (Krieger-Brauer

FIGURE 5: ^1H NMR spin-echo spectra of K_2EDTA-loaded ghosts after 6- (A) and 22-min (B) incubation with 10 mM $Ca(NO_3)_2$. The sample in (B) was made 8 μM in A23187, and the spectrum was recorded after a further 8 min of incubation (C). In spectrum C, the region to the high field of 2.8 ppm is obscured by Me_2SO, used as the solvent for A23187.

& Gratzl) and chromaffin granule ghosts (Phillips, 1981). NMR spectra obtained 10 and 22 min after Ca^{2+} addition to ghosts with no transmembrane Na^+ gradient showed no evidence of Ca^{2+} transport (Figure 5A,B), in contrast to results from incubations carried out in the presence of an opposing Na^+ gradient (Figure 4). Ca^{2+} uptake was subsequently observed by NMR in the K_2 EDTA-containing ghost suspension following the addition of the Ca^{2+} ionophore A23187 (Figure 5C,D).

Discussion

The purpose of the present work was to determine whether Ca^{2+} transport across the chromaffin granule membrane is directly linked energetically to a transmembrane proton gradient. For this purpose, we have monitored pH changes within the lumen of chromaffin granule ghosts during Ca^{2+} entry and complexation with intravesicular EDTA. These experiments were conducted in the presence of luminal Na^+, since Phillips (1981) and Krieger-Brauer & Gratzl (1981) have proposed Na^+/Ca^{2+} antiport activity in this membrane, and since chromaffin granules contain fairly high endogenous levels of Na^+ (Phillips et al., 1977).

The observed pH changes in the chromaffin granule lumen may reflect, in addition to the nonequilibrium contribution due to deprotonation of Ca·EDTA, a pH gradient due to the altered Donnan equilibrium produced by Ca^{2+} entry. Several experimental observations indicate that the attainment of equilibrium with respect to passive proton movements is slow on the time scale of the NMR experiments. In NMR experiments monitoring acidification of the lumen by means of ATP-induced proton translocation, as shown in Figure 2 and 3, or Ca^{2+} uptake, as shown in Figure 4, the nonequilibrium pH gradient persisted for tens of minutes following completion of the reaction. The gradient was collapsed by added nigericin as detected by narrowing of unbound peak widths and concurrent shift to higher field. The maintenance of a nonequilibrium pH gradient over this time period is consistent with

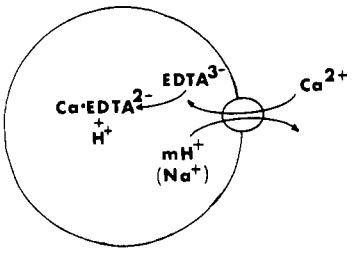

FIGURE 6: Stoichiometry of proton release and proton (sodium) extrusion during Ca^{2+} entry into chromaffin granule ghosts.

previous observations concerning the proton conductivity of the chromaffin granule membrane (Johnson & Scarpa, 1976; Phillips, 1976), which is extremely low. Johnson and Scarpa, who reported an upper limit of the proton conductivity of the membrane of ≤ 0.09 μmol of $H^+ \cdot s^{-1} \cdot (pH\ unit)^{-1} \cdot (g\ of\ protein)^{-1}$, observed no significant decrease in an imposed pH difference across membranes of intact chromaffin granules over a period of several minutes. Phillips found a similar result (i.e., a passive H^+ permeance too low to be measured) in chromaffin granule ghosts. This value of the limited proton conductivity, in conjunction with the estimated buffering capacity of the lumen in our ghost preparations [approximately 1.8 mmol of H^+/(g of protein)], indicates that attainment of the altered Donnan equilibrium by passive proton conductance requires a period >28 h.

The stoichiometry of Ca^{2+}, H^+, and Na^+ transport is indicated diagrammatically in Figure 6. When Ca^{2+} enters the lumen, the cation is chelated by EDTA, which loses a proton. If Ca^{2+} transport were obligatorily coupled to an outward movement of protons, then the proton liberated by Ca·EDTA would not result in net acidification of the lumen. The fact that acidification is observed upon Ca^{2+} entry indicates that the Ca^{2+} uptake process proceeds without concurrent proton extrusion (i.e., $m \leq 0$). These results indicate that the endogenous proton gradient of intact chromaffin granules is not the immediate driving force for Ca^{2+} uptake, although an indirect coupling to the protonmotive force, possibly via a Na^+ ion gradient, is not excluded.

Registry No. Ca, 7440-70-2; H^+, 12408-02-5; Na, 7440-23-5; Na_2EDTA, 139-33-3; EDTA, 60-00-4; Ca·EDTA, 12264-18-5.

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Mutagenesis by N^4-Aminocytidine: Induction of AT to GC Transition and Its Molecular Mechanism†

Kazuo Negishi, Mitsuko Takahashi, Yasuhiro Yamashita, Masahiko Nishizawa, and Hikoya Hayatsu*
Faculty of Pharmaceutical Sciences, Okayama University, Tsushima, Okayama 700, Japan
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ABSTRACT: N^4-Aminocytidine is a potent mutagen toward *Escherichia coli* and *Salmonella typhimurium*. It induced reversion of an amber mutant of ϕX174 phage (am3) to the wild type. This reversion was shown to be exclusively due to the AT to GC transition. It is likely that N^4-aminocytidine is metabolized within the bacterial cells into N^4-aminodeoxycytidine 5′-triphosphate and this nucleotide is incorporated into DNA during the multiplication of the cells and the phages, thereby causing base-pair transitions. The molecular basis for this erroneous replication was obtained in studies of in vitro incorporation of N^4-aminodeoxycytidine 5′-triphosphate into polynucleotides catalyzed by the *E. coli* DNA polymerase I large fragment. The results have shown that this cytosine analogue can be efficiently incorporated as a substitute of cytosine and that it can also be incorporated as a substitute of thymine. The ratio in the rate of the N^4-aminocytosine nucleotide incorporation to that of natural nucleotide incorporation was 1/2 to cytosine and 1/30 to thymine. Furthermore, the N^4-aminocytosine residues in the polynucleotide templates can be read by the enzyme as efficiently as cytosines, and guanines were incorporated opposite to them.

Recently, we have found that N^4-aminocytidine can efficiently induce base-change mutations in bacteria and in phages (Negishi et al., 1983). Its high activity is exceptional for a nucleoside analogue and is comparable to that of *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine, one of the most potent mutagens. The activity is more than 1000 times stronger than that of 2-aminopurine in the reversion of *Salmonella typhimurium* TA100 as well as in the reversion of *Escherichia coli* WP2 *trp* (ocher). Thus, the numbers of revertants formed per nanomole of reagent (calculated from the linear dose–responses) were, N^4-aminocytidine/2-aminopurine/*N*-methyl-*N*′-nitro-*N*-nitrosoguanidine, in *S. typhimurium* TA100 60/0.02/300 and in *E. coli* WP2 uvr 160/0.012/10 (Negishi et al., 1983; unpublished work).

Probably, within the cells N^4-aminocytidine is converted to $dC^{am}TP$[1] and incorporated into DNA. The principal tautomeric structure of N^4-aminocytosine is the amino form (Brown et al., 1968; Takayanagi et al., 1980), and Brown et al. (1968) have estimated that about $^1/_{30}$ of the molecules is present in the imino form. A simple mechanism for the N^4-aminocytosine-induced mutagenesis would be that the imino form of N^4-aminocytosine pairs with adenine, while the major amino form pairs with guanine (Figure 1). Thus, during one replicational cycle, $dC^{am}TP$ is incorporated into DNA mostly as a substitute of dCTP, but it can also be incorporated to a certain extent as a substitute of dTTP (see step 1 in Figure 1). In the next cycle of replication, either dGTP or dATP can be incorporated opposite N^4-aminocytosine on the template DNA (step 2). This scheme is analogous to the classical one proposed by Freese (1959) for the base analogue mutagenesis.

The mechanism in Figure 1 predicts that N^4-aminocytidine should induce both the AT to GC and the GC to AT transitions and that no transversions should be caused. In the present work, we analyzed the mutational specificity in the N^4-aminocytidine-induced reversion of ϕX174 am3 phage and studied the in vitro utilization of $dC^{am}TP$ in the polynucleotide synthesis catalyzed by *E. coli* DNA polymerase I large fragment. The results obtained by sequencing the revertant phage DNAs have shown that a AT to GC transition took place in the am3 site. Furthermore, the study on the in vitro DNA synthesis gave strong support for the mechanism as illustrated in Figure 1.

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[1] Abbreviations: $dC^{am}TP$, N^4-amino-2′-deoxycytidine 5′-triphosphate; $dC^{oh}TP$, N^4-hydroxy-2′-deoxycytidine 5′-triphosphate; $imdC^{am}TP$ (and $imdC^{am}MP$), 6-(2′-deoxy-β-D-ribofuranosyl)-5-oxo-5,6-dihydro-*s*-triazolo[4,3-*c*]pyrimidine 5′-triphosphate (and 5′-monophosphate); HPLC, high-pressure liquid chromatography; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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