Equilibrium Thermodynamics, Formation, and Dissociation Kinetics of Trivalent Iron and Gallium Complexes of Triazacyclononane-Triphosphinate (TRAP) Chelators: Unraveling the Foundations of Highly Selective Ga-68 Labeling

Adrienn Vágner, Attila Forgács, Ernő Brücher, Imre Tóth, Alessandro Maiocchi, Alexander Wurzer, Hans-Jürgen Wester, Johannes Notni and Zsolt Baranyai

In order to rationalize the influence of Fe\textsuperscript{III} contamination on labeling with the \(^{68}\text{Ga}\) eluted from \(^{68}\text{Ge}/^{68}\text{Ga}\)-generator, a detailed investigation was carried out on the equilibrium properties, formation and dissociation kinetics of Ga\textsuperscript{III}- and Fe\textsuperscript{III}-complexes of 1,4,7-triazacyclononane-1,4,7-tris(methylene[2-carboxyethylphosphinic acid]) (H\textsubscript{3}TRAP). The stability and protonation constants of the [Fe(TRAP)]\textsuperscript{3-} complex were determined by pH-potentiometry and spectrophotometry by following the competition reaction between the TRAP ligand and benzhydroxamic acid (0.15 M NaNO\textsubscript{3}, 25°C). The formation rates of [Fe(TRAP)] and [Ga(TRAP)] complexes were determined by spectrophotometry and \(^{31}\text{P}\)-NMR spectroscopy in the pH range 4.5–6.5 in the presence of 5–40 fold H\textsubscript{x}TRAP\textsubscript{(x–6)} excess (x = 1 and 2, 0.15 M NaNO\textsubscript{3}, 25°C). The kinetic inertness of [Fe(TRAP)]\textsuperscript{3-} and [Ga(TRAP)]\textsuperscript{3-} was examined by the trans-chelation reactions with 10 to 20-fold excess of H\textsubscript{x}HBED\textsuperscript{(x–4)} ligand by spectrophotometry at 25°C in 0.15 M NaCl (x = 0, 1 and 2). The stability constant of [Fe(TRAP)]\textsuperscript{3-} (log\textsubscript{K}\textsubscript{FeL} = 26.7) is very similar to that of [Ga(TRAP)]\textsuperscript{3-} (log\textsubscript{K}\textsubscript{GaL} = 26.2). The rates of ligand exchange reaction of [Fe(TRAP)]\textsuperscript{3-} and [Ga(TRAP)]\textsuperscript{3-} with H\textsubscript{x}HBED\textsuperscript{(x–4)} are similar. The reactions take place quite slowly via spontaneous dissociation of [M(TRAP)]\textsuperscript{3-}, [M(TRAP)OH]\textsuperscript{4-} and [M(TRAP)(OH)\textsubscript{2}]\textsuperscript{5-} species. Dissociation half-lives (t\textsubscript{1/2}) of [Fe(TRAP)]\textsuperscript{3-} and [Ga(TRAP)]\textsuperscript{3-} complexes are 1.1 × 10\textsuperscript{5} and 1.4 × 10\textsuperscript{5} h at pH = 7.4 and 25°C. The formation reactions of [Fe(TRAP)]\textsuperscript{3-} and [Ga(TRAP)]\textsuperscript{3-} are also slow due to the formation of the unusually stable monoprotonated [*M(HTRAP)]\textsuperscript{2-} intermediates [log\textsubscript{K}\textsubscript{Ga(HL)} = 10.4 and [log\textsubscript{K}\textsubscript{Fe(HL)} = 9.9], which are much more stable than the [*Ga(HNOTA)]\textsuperscript{+} intermediate [log\textsubscript{K}\textsubscript{Ga(HL)} = 4.2]. Deprotonation...
INTRODUCTION

Due to the wealth of obtainable information resulting in a high diagnostic value, medical imaging plays an ever-increasing role in modern personalized healthcare. In this context, radionuclide based imaging modalities which exploit George Hevesy’s tracer principle (Levi, 1976) allow for unique functional diagnostics, because they enable monitoring of biological processes without significant interference with the investigated subject owing to minuscule amounts of administered active compound. Although the majority of nuclear imaging procedures (estimated > 85%) still are scintigraphic or single photon emission computed tomography (SPECT) scans relying on the gamma-emitter $^{99m}$Tc, recent times have seen a strong surge in positron emission tomography (PET), following introduction of scanners capable of simultaneous functional and morphological imaging utilizing PET and computed tomography (CT) in 2001 (Beyer et al., 2000). While most PET investigations rely on the positron emitter $^{18}$F (more precisely, on the radiofluorinated glucose derivative $[^{18}$F]$2$-fluoro-2-deoxy-D-glucose), some positron-emitting metal ion radionuclides have also received considerable attention in recent times (Wadas et al., 2010). Among these, $^{68}$Ga has arguably the highest value for preclinical and translational studies (Notni and Wester, 2018), mainly because it is obtained for a low price per dose from radionuclide generators. These small benchtop devices, which act as cyclotron-independent continuous on-site nuclide sources, contain $^{68}$Ge adsorbed on an inorganic matrix, such as SnO$_2$ or TiO$_2$, while decay of $^{68}$Ge produces $^{68}$Ga$^{III}$ which can be eluted with dilute HCl (Notni, 2012; Rösch, 2013). Notably, such eluate frequently contains small amounts of impurities originating from the sorbent (Simecek et al., 2013), such as Ti$^{IV}$ but also Fe$^{III}$, Cu$^{II}$, and Zn$^{II}$, or Al$^{III}$ in form of their aqua or chlorido complexes.

$^{68}$Ga-labeling of biomolecules usually requires prior decoration with a suitable multidentate ligand capable of binding the $^{68}$Ga$^{III}$ ion into a kinetically inert complex (Wadas et al., 2010) and a plethora of ligands have been proposed for this purpose (Frank and Patrick, 2010; Velikyan, 2011). Against the background of aforementioned metal ion impurities in the generator eluate, an investigation of the radionuclide complexation efficiency of certain macrocycle-based chelators, among them TRAP (Notni et al., 2014) and NOTA (Mariko and Susumu, 1977; Scheme 1) pointed at a markedly different influence of non-Ga$^{III}$ metal ions present in the $^{68}$Ga$^{III}$ solutions used for radiolabeling (Simecek et al., 2013). In particular, TRAP was shown to tolerate much higher concentrations of Zn$^{II}$, Cu$^{II}$, and Fe$^{III}$. Although highly similar structural features of [Fe(H$_3$TRAP)]$^+$ and [Ga(H$_3$TRAP)]$^+$ at close relation of both systems (Notni et al., 2010), it was found that even a threefold stoichiometric excess of Fe$^{III}$ over TRAP or its mono-conjugable congener NOPO (Simecek et al., 2014) did not result in a significant decrease of $^{68}$Ga incorporation, whereas labeling of NOTA was almost completely inhibited. Particularly in view of the known similarity of Fe$^{III}$ and Ga$^{III}$, this discrepancy sheds a light on the mechanisms governing the superior $^{68}$Ga labeling properties of 1,4,7-triazacyclononanones bearing (methylene)phosphinic acid N-substituents (Notni et al., 2011). In order to gain a more detailed understanding, thermodynamics as well as formation and dissociation kinetic studies were performed for Ga$^{III}$- and Fe$^{III}$-complexes formed with TRAP and NOTA.

MATERIALS AND METHODS

Materials

The chemicals used for the experiments were of the highest analytical grade. Ga(NO$_3$)$_3$ and Fe(NO$_3$)$_3$ were prepared by
dissolving Ga$_2$O$_3$ (99.9%, Fluka) and Fe$_2$O$_3$ (99.9% Fluka) in 6 M HNO$_3$ and evaporating of the excess acid. The solid Ga(NO$_3$)$_3$ and Fe(NO$_3$)$_3$ were dissolved in 0.1 M HNO$_3$ solution. The concentration of the Ga(NO$_3$)$_3$ and Fe(NO$_3$)$_3$ solutions were determined by complexometry with the use of standardized Na$_2$H$_2$EDTA in excess. The excess of the Na$_2$H$_2$EDTA was measured with standardized ZnCl$_2$ solution and xylenol orange as indicator. The H$^+$ concentration of the Ga(NO$_3$)$_3$ and Fe(NO$_3$)$_3$ solutions was determined by pH potentiometric titration in the presence of Na$_2$H$_2$EDTA excess. The concentration of the H$_2$TRAP, H$_2$HBED, benzohydroxamic acid (HBha) and H$_3$NOTA (provided by Prof. Petr Hermann, Department of Inorganic Chemistry, Faculty of Science, Charles University, Prague, Czech Republic) was determined by pH-potentiometric titration in the presence and absence of a large (40-fold) excess of CaCl$_2$. All the measurements were made at constant ionic strength maintained by 0.15 M NaNO$_3$ or NaCl at 25°C.

**Equilibrium Studies**

For determining the protonation constants of H$_2$TRAP and H$_2$NOTA ligands three parallel pH-potentiometric titration were made with 0.2 M NaOH in 0.002 M ligand solutions.

Stability constant of [Fe(Bha)]$^{2+}$ complex was determined by spectrophotometry, studying the Fe$^{III}$-HBha systems at the absorption band of Fe$^{III}$-complex over the wavelength range of 400–800 nm in two sets of experiments. Individual samples were prepared in the first series in which the concentrations of Fe$^{III}$ and HBha was constant 0.2 and 2.0 mM, while that of the H$^+$ was varied between 0.04 and 1.0 mM (eight samples, Figure S1). The H$^+$ concentration in the samples was adjusted by addition of calculated amounts of 2.0 M HNO$_3$. The ionic strength was constant in the samples with [H$^+$]=0.15 M ([H$^+$]+[Na$^+$] = 0.15 M). Samples were kept at 25°C for a week. Absorbance values were determined at 11 wavelengths (400, 415, 430, 445, 460, 475, 490, 505, 520, 535, and 550 nm). In the second set, spectrophotometric titrations were done with samples containing HBha ligand in 2.0 mM concentration, whereas the concentration of Fe$^{III}$ was varied between 0.1–0.3 mM (Figures S2–S4). The pH of the samples was adjusted using concentrated NaOH and HNO$_3$ solutions in the pH range 1.7–11.0 (0.15 M NaNO$_3$ and 25°C). For calculation of the equilibrium constants, the best fit of the absorbance–pH data was obtained by assuming formation of [Fe(Bha)]$^{2+}$, [Fe(Bha)$_2$]$^{3+}$, [Fe(Bha)$_3$], and [Fe(Bha)$_2$(OH)$_2$]$^{-}$ species (Figure S5). The molar absorptivity of [Fe(Bha)]$^{2+}$, [Fe(Bha)$_2$]$^{3+}$, [Fe(Bha)$_3$] and [Fe(Bha)$_2$(OH)$_2$]$^{-}$ species were also determined at the same 11 wavelengths in these experiments (Figure S6).

The stability constant of the [Fe(TRAP)]$^{3-}$ complex has been determined by spectrophotometry, using competition reactions between HTRAP$^{5-}$ and Bha$^-$ for Fe$^{III}$ at pH = 10.0. Concentration of [Fe(TRAP)]$^{3-}$ was 0.2 mM, while that of HBha was varied between 0.0 and 1.5 mM (6 samples). The samples were kept at 25°C for 2 weeks. Absorbance values of the Fe$^{III}$-HTRAP$^{5-}$-Bha$^-$ systems were determined at 11 wavelengths (400, 415, 430, 445, 460, 475, 490, 505, 520, 535, and 550 nm). The molar absorptivities of [Fe(TRAP)]$^{3-}$ and [Fe(TRAP)OH]$^{4-}$ in equilibrium solutions were determined by recording the absorption spectra of 0.1, 0.2, and 0.3 mM solution of [Fe(TRAP)]$^{3-}$ in the pH range 6.0–12.0. The molar absorptivity of [Fe(Bha)$_2$(OH)$_2$]$^{3-}$ species was determined in the separate experiments. Absorbance and pH values were determined in the samples after equilibration (the time needed to reach the equilibria was determined by spectrophotometry). Spectrophotometric measurements were done using 1.0 cm cells with a Cary 1E spectrophotometer at 25°C. Protonation constants of the Fe$^{III}$ complex formed with TRAP$^{5-}$ were determined by direct pH-potentiometric titration at 1:1 metal to ligand ratios (both concentrations were 0.002 M). For calculation of the logK$_{MHL}$ values, the mL base–pH data used were measured in the pH range 1.7–12.0.

For pH measurements and titrations, a Metrohm 785 DMP Titriso titration workstation and a Metrohm-6.0233.100 combined electrode were used. Equilibrium measurements were carried out at a constant ionic strength (0.15 M NaNO$_3$ or NaCl) in 6 mL samples at 25°C. Solutions were stirred and continuously purged with N$_2$. Titrations were performed in a pH range of 1.7–12.0. KH-phthalate (pH = 4.005) and borax (pH = 9.177) buffers were used to calibrate the pH meter. For calculation of [H$^+$] from measured pH values, the method proposed by Irving et al. was used (Irving et al., 1967). A 0.01 M HNO$_3$ or HCl solution was titrated with the standardized NaOH solution in the presence of 0.15 M NaNO$_3$ or NaCl. Differences between the measured (pH$_{read}$) and calculated pH (–log[H$^+$]) values were used to obtain the equilibrium H$^+$ concentration from the pH values, measured in the titration experiments. For equilibrium calculations, the stoichiometric water ionic product (pK$_w$) is also needed to calculate [H$^+$] values in basic conditions. The V$_{NaOH}$–pH$_{read}$ data pairs of the HNO$_3$–NaOH or HCl–NaOH titration obtained in the pH range 10.5–12.0 have been used to calculate the pK$_w$ value (pK$_w$ = 13.84). For calculation of the equilibrium constants, the program PSEQUAD (Zekany and Nagypré, 1985) was used. The standard deviation (SD) of the equilibrium parameters calculated by the program PSEQUAD is defined by Equation (1)

$$SD = \sqrt{\frac{\sum_{i=1}^{N} (F_{\text{res}} - \sum_{j=1}^{m} J_{\text{res}}) \cdot (J_{\text{res}} - \frac{1}{T})^{-1}}{N - m}} \times \sqrt{\sum_{i=1}^{N} (F_{\text{res}} - \sum_{j=1}^{m} J_{\text{res}}) \cdot (J_{\text{res}} - \frac{1}{T})^{-1}}$$

where res, N, m, J and J$^T$ are the residual, number of fitted data, number of refined parameters, Jacobian matrix and the transpose of Jacobian matrix, respectively.

**Kinetic Studies**

Formation Kinetics of [Fe(TRAP)] and [Ga(TRAP)]

Formation rates of [Fe(TRAP)] were studied by spectrophotometry at 260 nm in the pH range of about 4.5–6.5. Kinetic studies were carried out with Cary 1E and Cary 100 Bio spectrophotometers, using cell holders thermostated to 25°C. The pre-thermostated solutions were mixed in tandem cells (l = 0.874 cm). Formation of Fe$^{III}$ complexes were studied in the presence of a 5- to 40-fold ligand excess in order to maintain pseudo-first-order conditions ([Fe$^{III}$] = 0.1 mM).
Pseudo-first-order rate constants \((k = k_{\text{obs}})\) were calculated by fitting the absorbance values to the equation:

\[
A_t = (A_0 - A_e)e^{(-kt)} + A_e
\]  

(2)

where \(A_0\), \(A_e\), and \(A_t\) are the absorbance values at the start \((t = 0\ s)\), at equilibrium and at the time \(t\) of the reaction, respectively. Formation of [Ga(TRAP)]\(^{3+}\) was monitored by \(^{31}\)P-NMR spectroscopy on the signal of the forming Ga(TRAP) complex. \(^{31}\)P-NMR spectra were recorded by a Bruker DRX 400 spectrometer \((31\text{P, 161.97 MHz, 9.4 T})\) equipped with Bruker VT-1000 thermocoupler, using a 5 mm broad band probe. Kinetic experiments were performed at a constant temperature of 25.0°C. The formation rates were studied in the pH range of about 4.5–6.3. For these experiments, Ga(NO\(_3\))\(_3\) and H\(_6\)TRAP solutions were prepared in H\(_2\)O (a capillary with D\(_2\)O was used for lock). In all experiments, the concentration of Ga\(^{3+}\) was 1 mM, while that of the H\(_6\)TRAP was varied between 5 and 30 fold excess in order to maintain pseudo-first-order conditions. Pseudo-first-order rate constants \((k = k_{\text{obs}})\) were calculated by fitting the integral signal values to the Equation (2). The ionic strength of the solutions was kept constant at 0.15 M with NaNO\(_3\). To keep the pH values constant, N-methylpiperazine (pH range of 4.1–5.2) and piperezine (pH range of 4.7–6.6) buffers \((0.01\ M)\) were used.

**Dissociation Kinetics of Fe(TRAP) and Ga(TRAP)**

The rates of the ligand exchange reactions of Fe(TRAP) and Ga(TRAP) with H\(_6\)HBED\(^{x-}\) \((x = 0.1, 2)\) ligand were studied by following the formation of [Fe(HBED)]\(^{−}\) and [Ga(HBED)]\(^{−}\) complexes by spectrophotometry at 470 nm and 290 nm, respectively. All experiments were performed in the presence of 10- and 20-fold excess of H\(_6\)HBED\(^{x-}\) \((x = 1\) and 2\) in order to maintain pseudo-first order kinetic conditions \((|\text{Fe(TRAP)}| = |\text{Ga(TRAP)}| = 0.2\ mM)\). The pseudo-first-order rate constants \((k = k_{\text{obs}})\) were calculated by fitting the absorbance values to the Equation (2). Kinetic studies were performed with Cary 1E and Cary 100 Bio spectrophotometers, using cell holders thermostatd to 25°C. The pre-thermostated solutions were mixed in tandem cells \((I = 0.874\ cm)\). The ionic strength of the solutions was kept constant at 0.15 M with NaCl.

The ligand exchange reactions were followed at 25°C in the pH range 9.0–14.0. The OH\(^−\) concentration at pH > 12 was adjusted by addition of calculated amounts of 4.0 M NaOH solution. Buffers were not used to keep the pH constant due to the high buffer capacity of the H\(_6\)HBED\(^{x-}\) \((x = 1\) and 2\) excess at pH < 12. Calculation of the kinetic parameters was performed with the Micromath Scientist computer program (version 2.0, Salt Lake City, UT, USA).

**RESULTS AND DISCUSSION**

**Solution Thermodynamics**

Protonation equilibria of the TRAP\(^{6−}\), NOTA\(^{3−}\) and Bha\(^{−}\)ligands were studied by pH-potentiometry. The protonation constants \((\log K_i^H)\) of ligands defined by Equation (3) are listed in Table 1 (standard deviations are shown in parentheses). The charges of ligands and complexes will be indicated when it is necessary.

\[
K_i^H = \frac{[H_iL]}{[H_{i-1}L][H^+]}
\]

(3)

The protonation schemes of TRAP\(^{6−}\) and NOTA\(^{3−}\) ligands were well characterized by both spectroscopic and potentiometric methods (Bevilacqua et al., 1987; Geraldes et al., 1991; Nottni et al., 2010). These studies reveal that the first and second protonations occur at two ring nitrogen atoms, whereas the third, fourth and fifth protonations occur at the carboxylate groups of NOTA\(^{3−}\) and TRAP\(^{6−}\). The sixth proton of the TRAP\(^{6−}\) ligand binds on the phosphinate oxygen atom. Interestingly, not all phosphate groups are protonated, even under very acidic conditions \((\text{pH} < 1)\), which is why they are still able to coordinate to metal ions. A comparison of protonation constants of TRAP\(^{6−}\) and NOTA\(^{3−}\) indicates that log\(K_i^H\) value of TRAP\(^{6−}\) is significantly lower than that of NOTA\(^{3−}\) (Table 1). The lower first protonation constant of TRAP\(^{6−}\) can be attributed to formation of a weaker H-bond between the protonated ring nitrogen and the phosphate oxygens than that formed between the protonated ring nitrogen and the carboxylate oxygens in HNOTA\(^{3−}\). Comparison of the protonation constants obtained in 0.15 M NaNO\(_3\) or NaCl, 0.1 M KCl and 0.1 M Me\(_4\)NCl solutions indicates that the log\(K_i^H\) values of TRAP\(^{6−}\) are independent of the ionic strength, whereas the log\(K_i^H\) value of NOTA\(^{3−}\) is significantly lower in the presence of K\(^+\) and Na\(^+\) ions, which can be attributed to formation of [K(NOTA)]\(^{2−}\) and [Na(NOTA)]\(^{2−}\) complexes. Total basicity of ligands (Σlog\(K_i^H\), Table 1) generally correlates with the stability constants \((K_{ML})\) of their metal complexes. (For the calculation of Σlog\(K_i^H\) value of TRAP\(^{6−}\), the log\(K_i^H\) values of the carboxylate groups were not considered because they do not participate in the coordination of metal ions). The Σlog\(K_i^H\) values (Table 1) show that the total basicity of TRAP\(^{6−}\) is significantly lower than that of NOTA\(^{3−}\) because of the lower protonation constant of the ring nitrogen (log\(K_i^H\)) and phosphate oxygen atoms of the TRAP\(^{6−}\) ligand. Therefore, lower stability constants should be expected for the TRAP\(^{6−}\) complexes than those of NOTA\(^{3−}\) complexes.

Stability and protonation constants of TRAP\(^{6−}\) and NOTA\(^{3−}\) complexes formed with Fe\(^{3+}\) were determined by pH-potentiometry and UV/Vis spectrophotometry. The stability and protonation constants of the metal complexes formed with the TRAP\(^{6−}\) and NOTA\(^{3−}\) ligands listed in Table 2 are defined by Equations (4–6):

\[
\begin{align*}
M^{3+} + L & \rightleftharpoons ML \\
K_{ML} &= \frac{[ML]}{[M][L]} \\
M(\text{OH})^{-} + H^{+} & \rightleftharpoons M(\text{OH})H^{+} \\
K_{M(\text{OH})H^{+}} &= \frac{[M(\text{OH})H^{+}]}{[M(\text{OH})][H^{+}]} \quad (5) \\
M(\text{L})OH + H^{+} & \rightleftharpoons ML \\
K_{M(\text{L})OH} &= \frac{[ML]}{[M(\text{L})OH][H^{+}]} \quad (6)
\end{align*}
\]
TABLE 1 | Protonation constants of TRAP$^{3-}$, NOTA$^{3-}$, and Bha$^{−}$ ligands (25°C).

|        | I       | log$K^H_{1}$ | log$K^H_{2}$ | log$K^H_{3}$ | log$K^H_{4}$ | log$K^H_{5}$ | log$K^H_{6}$ | $\Sigma$log$K^H_i$ |
|--------|---------|-------------|-------------|-------------|-------------|-------------|-------------|-----------------|
| TRAP$^{3-}$ | 0.15 M NaNO$_3$ | 11.60(2) | 5.39(2) | 4.42(2) | 4.19(3) | 3.46(3) | 1.60(2) | 18.59 $^g$ |
|         | 0.15 M NaCl$^b$ | 11.74 | 5.46 | 4.80 | 4.16 | 3.49 | 1.50 | 18.70 $^g$ |
|         | 0.1 M Me$_2$NCl$^b$ | 11.48 | 5.44 | 4.84 | 4.23 | 3.45 | 1.66 | 18.58 $^g$ |
| NOTA$^{3-}$ | 0.15 M NaNO$_3$ | 11.94(2) | 5.71(3) | 3.14(3) | 1.60(2) | – | – | 22.39 |
|         | 0.15 M NaCl$^b$ | 12.16 | 5.75 | 3.18 | 1.90 | – | – | 22.99 |
|         | 0.1 M KCl$^c$ | 11.98 | 5.65 | 3.18 | – | – | – | 24.09 |
|         | 0.1 M Me$_2$NCl$^c$ | 13.17 | 5.74 | 3.22 | 1.96 | – | – | – |
| Bha$^{−}$ | 0.15 M NaNO$_3$ | 8.53(3) | – | – | – | – | – | – |
|         | 0.2 M KCl$^d$ | 8.69 | – | – | – | – | – | – |

$^a$Ref. [Baranyai et al., 2013]; $^b$Ref. [Notni et al., 2010]; $^c$Ref. [Clarke and Martell, 1991]; $^d$Ref. [Farkas et al., 1998]; $^e$Total ligand basicity (Σlog$K^H_i$) characterizes the sum of basicity of donor atoms; $^f$The protonation constants of the acetate pendants (log$K^H_i$) of TRAP$^{3-}$ were not considered in the calculation of Σlog$K^H_i$ values.

TABLE 2 | Stability and protonation constants (logK) of Fe$^{III}$ and Ga$^{III}$-complexes formed with TRAP$^{3-}$, NOTA$^{3-}$, and Bha$^{−}$ ligand (25°C).

|       | Fe$^{III}$ | Ga$^{III}$ | Fe$^{III}$ | Ga$^{III}$ | Fe$^{III}$ |
|-------|-----------|-----------|-----------|-----------|-----------|
| TRAP$^{3-}$ | I | 0.15 M NaNO$_3$ | 0.1 M NaCl | 0.1 M Me$_2$NCl$^b$ | 0.15 M NaNO$_3$ | 0.2 M NaCl |
|        | ML | 26.73(8) | 26.24 | 28.3 | 29.60 | 10.80(2) | 11.08 |
|        | MHL | 5.07(2) | 5.18 | – | 0.9 | – | – |
|        | MH$_2$L | 4.34(2) | 4.55 | – | – | – | – |
|        | MH$_3$L | 3.20(2) | 3.77 | – | – | – | – |
|        | MH$_4$L | – | 0.7 | – | – | – | – |
|        | ML$_2$(OH) | 9.76(2) | 9.84 | 9.12(4)$^a$ | 9.83 | – | – |
|        | ML$_2$ | – | – | – | – | 9.03(2) | 10.12 |
|        | ML$_3$ | – | – | – | – | 7.41(3) | 7.60 |
|        | logK$_{Fe}$/(OH)$_2$ | – | – | – | – | 6.68(5) | – |

$^a$Ref. [Notni et al., 2010]; $^b$Ref. [Clarke and Martell, 1991]; $^c$Ref. [Simecek et al., 2012]; $^d$Ref. [Farkas et al., 1998]; $^e$In this work (0.15 M NaNO$_3$, 25°C).

wherein $i = 1, 2, 3$. Since the [Fe(TRAP)]$^{3−}$ and [Fe(NOTA)] complexes are highly stable, formation of Fe$^{III}$ complexes was practically completed at about pH < 2.0. Therefore, from the data obtained by pH-potentiometric titrations performed at 1:1 metal to ligand concentration ratio, only the protonation constants of the [Fe(TRAP)]$^{3−}$ and [Fe(NOTA)] complexes could be calculated. In order to determine the log$K_{FeL}$ value, we studied the competition reactions between HTRA$^{3−}$ and Bha$^{−}$ for Fe$^{III}$ [Equation (7)] by spectrophotometry in the wavelength range 400–800 nm. To calculate the stability constant of [Fe(TRAP)]$^{3−}$, the equilibrium constants characterizing the species formed in the Fe$^{III}$-HBha system have been determined from the data obtained by pH-potentiometric and spectrophotometric measurements (experimental detail and calculation procedures used for the characterization of Fe$^{III}$-HBha system are summarized in the Supplementary information).

\[\text{[Fe(TRAP)(OH)$_x$]}^{(3−x)} + \text{Bha}^{−} = \text{[Fe(Bha)$_2$(OH)$_2$]}^{−} + \text{HTRA$^{5−}$}\]

wherein $x = 0$ and 1. The pH of the samples was 10.0, when [Fe(TRAP)]$^{3−}$, [Fe(TRAP)OH]$^{4−}$ and [Fe(Bha)$_2$(OH)$_2$]$^{−}$ were formed. Some characteristic absorption spectra of Fe$^{III}$-HTRA$^{5−}$Bha$^{−}$ systems are shown in Figure 1.

The stability and protonation constants of [Fe(TRAP)]$^{3−}$ complex have been calculated by the combination of the pH-potentiometric data obtained by the titration of [Fe(TRAP)]$^{3−}$ complex with NaOH solution in the pH range 1.7–12.0 (Figure S7) with the spectrophotometric data acquired at pH = 10.0 in Fe$^{III}$-HTRA$^{5−}$-Bha$^{−}$ system (Figure 1). For calculation of the log$K_{FeL}$ value, protonation constants of Bha$^{−}$ (Table 1), the stability constant (Table 2) and the molar absorptivity of the [Fe(Bha)$_2$(OH)$_2$]$^{−}$ complex were used. Stability and protonation constants obtained for [Fe(TRAP)]$^{3−}$ are shown in Table 2.
Comparison of stability constants in Table 2 reveals that the logK_{ML} values of [Fe(TRAP)]^{3−} and [Ga(TRAP)]^{3−} complexes are essentially equal and 2−3 logK unit smaller than those of the corresponding NOTA^{3−} complexes. The higher stability constant of [Fe(NOTA)] and [Ga(NOTA)] complexes can be attributed to higher total basicity of NOTA^{3−}. The stability constant of [Fe(NOTA)] is about one logK unit lower than that of [Ga(NOTA)], which corresponds to a lower logK_{ML} value of NOTA^{3−} obtained in 0.1 M KCl solution. The triazaacyclononane macrocyclic ligands with carboxylate or phosphinate pendant arms show similar affinity to Fe^{3+} and Ga^{3+}, which is readily explained by the facts that Ga^{3+} and Fe^{3+} have similar ionic radii (0.62 Å and 0.65 Å, respectively), and share the same charge and preferred coordination number (CN = 6).

The species distribution diagram of the Fe^{III}−TRAP^{6−} system (Figure 2) shows that the Fe^{III} complex is fully formed even at pH < 2 in the form of a tri-protonated [Fe(H3L)] species. Upon rising the pH from 2.0 to 7.0, stepwise deprotonation results in consecutive formation of [Fe(H2L)]^{−} and [Fe(HL)]^{2−}. Since the protonation constants characterizing the formation of the [Fe(HL)]^{2−}, [Fe(H2L)]^{−} and [Fe(H3L)] species are very similar to the logK_{H1}, logK_{H2} and logK_{H3} values of the free TRAP^{6−} ligand, [Fe(TRAP)]^{3−} is protonated on the non-coordinating carboxylate pendant arms. According to the known solid state structures of [Fe(H3TRAP)], the coordination environment of Fe^{III} is characterized by the trigonal antiprismatic structure formed by the parallel ring-N₃ and phosphinate-O₃ planes, whereas the carboxylate groups are protonated and non-coordinated (the solid state structure of [GaH₃TRAP] complex is very similar to that of [Fe(H₃TRAP)]) (Notni et al., 2010). The [Fe(TRAP)]^{3−} complex predominates in the pH range 6.0−9.0. The pH-potentiometric titration data, obtained at pH > 8 for [Fe(TRAP)]^{3−}, indicate a base-consuming process, which can be attributed to substitution of one of the phosphinate oxygens with a OH^{−} ion in the coordination sphere of Fe^{III} upon formation of the [Fe(TRAP)OH]^{4−} species [Equation (6)]. Similar processes were also identified for [Ga(TRAP)]^{3−}, [Fe(NOTA)] (Figure S8 and Table 2) and [Ga(NOTA)] complexes (Notni et al., 2010; Simecek et al., 2012).

Formation Kinetics of Fe(TRAP) and Ga(TRAP) Complexes

The formation reactions between NOTA and various metals, such as lanthanide(III) ions (Ln^{III}) but also Ga^{III}, are typically slow at pH around 2.0−5.0 (Brucher and Sherry, 1990; Morfin and Toth, 2011). Since formation of Ln^{III} and Ga^{III} complexes of open-chain ligands is generally fast, the slow formation kinetics of the NOTA complexes can be attributed to the rigidity of the triaza-cyclononane macrocycle. Incorporation of Ln^{III} and Ga^{III}−ions into the preformed coordination cage of NOTA is slow because of formation of stable mono-protonated [Ln(HNOTA)]^{2+} and [Ga(HNOTA)]^{2+} intermediates, which has been confirmed earlier by spectrophotometry measurements (Brucher and Sherry, 1990) and ¹H NMR spectroscopy (Morfin and Toth, 2011). Stability constants of such intermediates have furthermore been determined from kinetic data obtained by spectrophotometry (Brucher and Sherry, 1990) and ¹H NMR spectroscopy (Morfin and Toth, 2011). In the intermediate, the proton is most likely attached to a macrocyclic nitrogen, and the electrostatic repulsion between the proton and a Ln^{III} or Ga^{III}−ion can inhibit fast entrance of the metal ion into the coordination cage. Formation rates of the [Ln(NOTA)] and [Ga(NOTA)] complexes are directly proportional to the OH^{−} concentration, meaning that a rate-determining OH^{−} assisted deprotonation and rearrangement of the monoprotonated intermediate is followed by entrance of the Ln^{III}, or Ga^{III}−ion into the N₂O₃ coordination cage of NOTA^{3−} (Brucher and Sherry, 1990; Morfin and Toth, 2011).

In the present work, formation kinetics of M(TRAP) complexes (M^{III} = Fe^{III} and Ga^{III}) have been studied by spectrophotometry on the absorption band of the forming Fe(TRAP) (λ = 260 nm) and by ³¹P-NMR spectroscopy following the integral value of the forming Ga(TRAP) complex in the pH range 4−6. UV-absorption as well as ³¹P-NMR spectra, recorded after mixing of solutions containing Fe(NO₃)₃ or Ga(NO₃)₃ with HTRAP^{5−} as functions of time, are shown in Figures S9, S10. For the reaction mixture of Fe^{III}−HTRAP^{5−} at pH = 6.0, the absorption band observed between λ = 245−320 nm (Figure S9) can be explained by the formation of the intermediate. The absorbance values in the λ = 250−280 nm range increase with time, allowing for the conclusion that the intermediate is transformed into the final [Fe(TRAP)]^{3−} in-cage complex. Formation of the intermediate in Ga^{III}−TRAP reactions mixtures was previously proven by ³¹P- and ⁷¹Ga-NMR spectroscopy (Notni et al., 2010). Based on the similarity of TRAP and NOTA, it can be assumed that protonation of the ring nitrogen below pH = 10.0 initially hampers the formation of in-cage TRAP complexes while the three carboxylate and three phosphinate oxygen atoms of HTRAP^{5−} can be coordinated to the metal ions to form a mono-protonated [M(HTRAP)]^{2−} intermediate, in which the Fe^{III} and Ga^{III}−ion is situated outside of the coordination cage. To complete the complex formation, the proton has to be removed from the ring nitrogen via a OH^{−}-assisted reaction, followed by the rearrangement of the intermediate to the final [Fe(TRAP)]^{3−} and [Ga(TRAP)]^{3−} complexes (Scheme 2).
The formation rates of [Fe(TRAP)]$^{3-}$ and [Ga(TRAP)]$^{3-}$ have been studied under pseudo-first-order conditions in the presence of high excess of H$_x$TRAP$^{(x-6)}$ ([Fe$^{III}$]) = 1.0 x 10$^{-4}$ M; [TRAP]$^t$ = 0.5–4.0 x 10$^{-3}$ M; [Ga$^{III}$] = 1.0 x 10$^{-3}$ M; [H$_x$TRAP]$^t$ = 5.0–30 x 10$^{-3}$ M, x = 1 and 2). Under such conditions the rate of formation reactions can be expressed by Equation (8).

$$\frac{d[ML]_t}{dt} = k_{obs}[M^{III}]_t$$  \hspace{1cm} (8)

wherein [ML]$_t$ is the concentration of the [Fe(TRAP)]$^{3-}$ and [Ga(TRAP)]$^{3-}$ complexes, [M$^{III}$]$_t$ is the total concentration of species containing the Fe$^{III}$ and Ga$^{III}$ ions not bound to the H$_x$TRAP$^{(x-6)}$ ligand, and $k_{obs}$ is a pseudo-first-order rate constant. As expected, the $k_{obs}$ vs. [H$_x$TRAP]$_t$ curves (Figures 3, 4) are saturation curves indicating the formation of the [*M(HTRAP)]$^{2-}$ intermediates characterized by the stability constant defined by Equation (9).

$$^{*}K_{M(HL)} = \frac{[^{*}M(HTRAP)]}{[M^{III}][HTRAP]}$$  \hspace{1cm} (9)

The rate-determining step of the reactions is the deprotonation and rearrangement of the [*M(HTRAP)]$^{2-}$ intermediates followed by the entrance of the metal ion into the coordination cage of the TRAP$^{6-}$ ligand:

$$\frac{d[ML]_t}{dt} = k_{obs}[M^{III}]_t = k_{i}[^{*}M(HTRAP)] = k_{i}[^{*}M(HTRAP)][M^{III}][HTRAP]$$  \hspace{1cm} (10)

wherein [*M(HTRAP)] is the concentration of [*M(HTRAP)]$^{2-}$ intermediate and $k_{i}$ is the rate constant characterizing the deprotonation and rearrangement of the intermediate to the [M(TRAP)]$^{3-}$ complex. In the pH range studied, the concentration of the non-complexed ligand ([TRAP]$_{free}$) can be expressed by Equation (11) using the protonation constants of TRAP$^{6-}$ ligand (Table 1).

$$[TRAP]_{free} = [HTRAP](1 + K_1^{III}[H^+] + K_2^{III}K_3^{III}[H^+]^2 + \ldots + K_2^{III}K_4^{III}K_5^{III}K_6^{III}[H^+]^5) = (1 + \alpha_1)[HTRAP]$$  \hspace{1cm} (11)

where $\alpha_1 = K_1^{III}[H^+] + K_2^{III}K_3^{III}[H^+]^2 + \ldots + K_2^{III}K_4^{III}K_5^{III}K_6^{III}[H^+]^5$. Under the conditions used in our experiments (pH = 4.0–6.0), hydrolysis of Fe$^{III}$ and Ga$^{III}$ may occur by formation of [M(OH)]$^{2+}$, [M(OH)$_2$]$^{+}$ and M(OH)$_3$ species, i.e., OH$^-$ ions may compete with H$_x$TRAP$^{(x-6)}$ for formation of [*M(HTRAP)]$^{2-}$ intermediate. Considering the hydrolysis of Fe$^{III}$ and Ga$^{III}$, the total metal ion concentration can be expressed

![Scheme 2](image-url)
By taking into account the stability constant of the [*M(HTRAP)]2− intermediate [Equation (9)] and the equilibrium constants characterizing the hydrolysis of FeIII and GaIII (βx = [M(OH)x][H+]x/[MIII], x = 1, 2, and 3), the total metal ion concentration can be expressed as follows:

$$[\text{M}^{\text{III}}] = [\text{M}^{\text{III}}] \left(1 + \frac{K_{\text{M(HTRAP)[TRAP]}^{\text{free}}}}{1 + \alpha_{\text{OH}}} + \frac{\beta_1^OH}{[H^+]^2} + \frac{\beta_2^OH}{[H^+]} + \frac{\beta_3^OH}{[H^+]^3}\right) = [\text{M}^{\text{III}}] \left(1 + \frac{K_{\text{M(HTRAP)[TRAP]}^{\text{free}}}}{1 + \alpha_{\text{OH}}} + \alpha_{\text{OH}}\right) (13)$$

wherein αOH = β2OH/[H+] + β3OH/[H+]2 + β3OH/[H+]3 (logβ2OH = −2.19; logβ3OH = −5.67 and logβ3OH = −12.0 for FeIII and logβ3OH = −2.97; logβ2OH = −5.92 and logβ3OH = −8.2 for GaIII ion; Baes and Mesmer, 1976). Considering the protonation constants of TRAP− (Table 1), the stability constant of the [*M(HTRAP)]2− intermediate [Equation (9)], the total concentration of the MIII ion [Equation (13)], the concentration of the non-complexed TRAPfree ligand [Equation (11) and Equation (10)], the pseudo-first order rate constant can be expressed by Equation (14).

$$k_{\text{obs}} = \frac{k_0^* K_{\text{M(HTRAP)[TRAP]}^{\text{free}}}}{1 + \frac{K_{\text{M(HTRAP)[TRAP]}^{\text{free}}}}{1 + \alpha_{\text{OH}}} + \alpha_{\text{OH}}} (14)$$

The pseudo-first-order rate constants determined at various pH and [TRAP] values (Figures 3, 4) were fitted to Equation (14) and the stability constant of the [*M(HTRAP)]2− intermediates [KM(HHL)] and the k0 rate constants were calculated.

The stability constants of the [*Fe(HTRAP)]2− and [*Ga(HTRAP)]2− intermediates [logK*M(HHL)] are 9.9 ± 0.1 and 10.4 ± 0.1, respectively. The logK*M(HHL) values of the [*Fe(HTRAP)]2− and [*Ga(HTRAP)]2− intermediates are significantly higher than those of the mono-protonated [*Ga(HNOTA)]+ (logK*G(HHL) = 4.2), (Morfin and Toth, 2011) [*Ce(HNOTA)]+ (logK*Ce(HHL) = 3.2), (Brucher and Sherry, 1990) [*Gd(HNOTA)]+ (logK*Gd(HHL) = 3.6) (Brucher and Sherry, 1990) and [*Er(HNOTA)]+ (logK*Er(HHL) = 3.8) (Brucher and Sherry, 1990) intermediates. In the [Fe(HTRAP)]2− and [*Ga(HTRAP)]2− intermediates, FeIII and GaIII are presumably coordinated by three carboxylate and three phosphate oxygen donor atoms, whereas the metal ions in [*M(HNOTA)]+ intermediates are coordinated by three carboxylate oxygen donor atoms, resulting in lower logK*M(HHL) values.

The calculated k0 rate constants obtained for formation of [Fe(TRA2P)]3− and [Ga(TRA2P)]3− complexes are shown in Figure 5 as functions of [OH−]. Kinetic data in Figure 5 show that the k0 values increase monotonously with increasing OH− concentration, while interception of linear extrapolations at the origin indicates that under our experimental conditions, deprotonation and transformation of the [*M(HTRAP)]2− intermediate to the final [M(TRA2P)]3− complex predominantly occurs by an OH−-catalyzed pathway. The kOH rate constants calculated from the slopes of the straight lines in Figure 5 are shown in Table 3.

Comparison of the kOH rate constants presented in Table 3 shows that the formation rates of [Ga(TRA2P)]3− and [Ga(NOTA)] complexes in this pathway are similar and about two orders of magnitude lower than those of [Ln(NOTA)] complexes. The results of the labeling experiments with the TRAP and NOTA chelates of 68GaIII at identical conditions (10 nM ligand, pH = 3.3 and 20 °C) shows that the formation rate of [68Ga(TRA2P)]3− surpasses that of [68Ga(NOTA)] (Notni et al., 2010). The faster formation of [68Ga(TRA2P)]3− can be explained by the higher stability [*KGa(HHL)] and consequently the higher concentration of the kinetically active [Ga(HTRAP)]2− intermediate that results in the more rapid formation of [68Ga(TRA2P)]3− in the same labeling condition. On the other hand, the formation rate of [Fe(TRA2P)]3− is about 3 times lower than that of Ga(TRA2P), which allows to perform selective labeling of TRAP with 68GaIII even in presence of FeIII contaminations in the eluate.

**Kinetic Inertness and Transchelation Reaction of Complexes**

In order to compare the kinetic inertness, the rates of transchelation reactions of Fe(TRA2P) and Ga(TRA2P) complexes with H2HBEDx− (x = 0, 1 and 2) ligand were studied because of the high stability of the [Fe(HBED)]− and [Ga(HBED)]− complexes [logKFe(HBED) = 39.01, logKGa(HBED) = 38.51, 0.1 M KCl, 25°C, (Ma et al., 1994)]. The transchelation reactions were followed by spectrophotometry on the absorption band of the forming [Fe(HBED)]− and [Ga(HBED)]− complexes in the pH ranges 11.0−14.0 and 9.0−12.0, respectively. The absorption spectra of the protonated HBBED3− and H2HBED2− ligands and [Ga(HBED)]− complex are different, whereas that of the deprotonated HBED2− ligand and [Ga(HBED)]− complex are very similar. Therefore, the transchelation reactions of [Ga(TRA2P)]3− with HBBED3− and H2HBED2− could be monitored by spectrophotometry only up to pH = 12.0 (HBED4−: logK4H = 12.57(4), logK3H = 11.41(3), logK2H = 8.22(5), logK1H = 4.73(6) and logK0H = 1.45(6), 0.15 M NaCl, 25°C). Some characteristic absorption spectra of [Fe(TRA2P)]3−, H2HBEDx− and [Ga(TRA2P)]3−−HxHBEDx− (x = 0, 1 and 2) reacting systems are shown in Figures S11, S12, respectively. The transchelation reactions can be described by Equation (15)

$$[\text{M(TRA2P)}]^{3−} + \text{H}_2\text{HBED}^{(x−4)} = [\text{M(HBED)}]^{−} + \text{H}_2\text{TRAP}^{(y−6)} + (x−y)\text{H}^{+} (15)$$

wherein MIII is FeIII or GaIII, x = 0, 1 and 2 and y = 0 and 1. The rates of the transchelation reactions have been studied in the presence of 10- and 20-fold excess of H2HBED(x−4), so a pseudo-first order kinetic model can be applied and the rates of reaction...
Equation (15) can be expressed by Equation (16):

\[
\frac{-d[M(\text{TRAP})]_t}{dt} = k_d[M(\text{TRAP})]_t
\]

where \( k_d \) is a pseudo-first-order rate constant, \([M(\text{TRAP})]_t\) is the total concentration of \([\text{Fe(TRA})P]^{3-}\) and \([\text{Ga(TRA})P]^{3-}\) complexes. The pseudo-first-order rate constants (\( k_d \)) characterizing the transchelation reactions of \([\text{Fe(TRA})P]^{3-}\) and \([\text{Ga(TRA})P]^{3-}\) with \( \text{H}_2\text{HBED}^{(x-4)} \) at different \(-\log[H^+]\) and \([-\log[\text{OH}^-]\) values are shown in Figure 6. The kinetic data presented in Figure 6 show that the \( k_d \) values are independent of the concentration of \( \text{H}_2\text{HBED}^{(x-4)} \) and increase with \(-\log[H^+]\) and \([-\log[\text{OH}^-]\), indicating that the rate-determining step of the transchelation reactions is the dissociation of the \([\text{Fe(TRA})P]^{3-}\) and \([\text{Ga(TRA})P]^{3-}\) complexes, followed by fast reaction of free Fe^{III} and Ga^{III} with \( \text{H}_2\text{HBED}^{(x-4)} \). The \( k_d \) values presented in Figure 6 show the similar behavior of \([\text{Fe(TRA})P]^{3-}\) and \([\text{Ga(TRA})P]^{3-}\) complexes in their transchelation reactions. The \( k_d \) vs. \(-\log[H^+]\) and \( k_d \) vs. \([-\log[\text{OH}^-]\) curves (Figure 6) obtained for \([\text{Ga(TRA})P]^{3-}\) and \([\text{Fe(TRA})P]^{3-}\) reach saturation of the \( k_d \) values at \([-\log[\text{OH}^-]\) > 0.015 M and \([-\log[H^+]\) > 1.0 M, respectively. Based on the species distribution of the Ga^{III}-TRAP^{6-} (Notni et al., 2010) and Fe^{III}-TRAP^{6-} (Figure 2) systems, the transchelation reaction of \([\text{Ga(TRA})P]^{3-}\) and \([\text{Fe(TRA})P]^{3-}\) with \( \text{H}_2\text{HBED}^{(x-4)} \) may occur by the spontaneous dissociation of \([M(\text{TRA})P]^{3-}\) (\( k_d \)) and \([M(\text{TRA})P]OH^{4-}\) species (\( M(L)OH^{[OH]} \)), whereas the \( pH \)-independent dissociation rate constant (\( k_d \)) of \([M(\text{TRA})P]^{3-}\) under basic conditions corresponds to formation \([K_{M(L)OH^{[OH]}} = \text{Equation (17)}\) and slow dissociation of the bis(hydroxo) \([M(\text{TRA})P(OH)]^{5-}\) intermediate.

\[
[M(\text{TRA})P(OH)]^{5-} + H^+ = [M(\text{TRA})P(OH)]^{4-} \quad \text{(17)}
\]

It can be assumed that in the \([M(\text{TRA})P(OH)]^{5-}\) intermediate, \( \text{TRA}^{6-} \) is coordinating via four donor atoms, whereas the remaining two coordination sites of Ga^{III} and Fe^{III} are occupied by two \( \text{OH}^- \) ions. Hence, a spontaneous dissociation of the \([M(\text{TRA})P(OH)]^{5-}\) intermediates is more probable, which is reflected by the \( M(L)OH^{[OH]} \) rate constants. The mechanisms of the transchelation reactions of \([\text{Fe(TRA})P]^{3-}\) and \([\text{Ga(TRA})P]^{3-}\) are summarized in Scheme 3.

By taking into account all possible pathways (Scheme 3), the dissociation rate of \([\text{Fe(TRA})P]^{3-}\) and \([\text{Ga(TRA})P]^{3-}\) can be expressed by Equation (18).

\[
\frac{-d[M(\text{TRA})P]_t}{dt} = \frac{k_d[M(\text{TRA})P]_t}{1 + K_{M(L)OH^{[OH]}}[H^+] + (K_{M(L)OH^{[OH]}}[H^+]^{-1})^{-1}}
\]

Considering the total concentrations of \([\text{Fe(TRA})P]^{3-}\) and \([\text{Ga(TRA})P]^{3-}\) (\( \text{ML}^{+} = [M(L)OH]+[M(L)(OH)_{2}] \)) and the protonation constants of \([M(L)OH]^{-}\) and \( K_{M(L)OH^{[OH]}} \) (Equation (6), Table 2) and \([M(L)OH]^{[OH]} \) intermediates (Equation (17)), the \( k_d \) pseudo-first-order rate constants presented in Figure 6 can be expressed by Equation (19).

\[
k_d = k_0 \frac{M(L)OH^{[OH]}[H^+] + M(L)OH^{[OH]}[H^+]^{-1} + K_{M(L)OH^{[OH]}}[H^+]^{-1}}{1 + K_{M(L)OH^{[OH]}}[H^+] + (K_{M(L)OH^{[OH]}}[H^+]^{-1})^{-1}}
\]

wherein \( k_0 \) are the rate constants characterizing the spontaneous dissociation of \([M(L)OH]^{[OH]} \) and \([M(L)OH]^{[OH]} \) complexes and \( K_{M(L)OH^{[OH]}} \) is the equilibrium constant characterizing the formation of the bis(hydroxo) \([M(\text{TRA})P(OH)]^{5-}\) intermediates.

The rate and protonation constants characterizing the transchelation reactions of \([\text{Fe(TRA})P]^{3-}\) and \([\text{Ga(TRA})P]^{3-}\) with \( \text{H}_2\text{HBED}^{(x-4)} \) have been calculated by fitting the \( k_d \) values presented in Figure 6 to the Equation (19), and the resulting values are shown in Table 3. We obtained a very low value with a large error for \( k_d \); therefore, the spontaneous dissociation of \([\text{Fe(TRA})P]^{3-}\) and \([\text{Ga(TRA})P]^{3-}\) is negligible under our experimental conditions. The \( M(L)OH^{[OH]} \) rate constants characterizing the spontaneous dissociation of \([\text{Fe(TRA})P(OH)]^{4-}\) and \([\text{Ga(TRA})P(OH)]^{4-}\) complexes are very similar, which indicates that the kinetic inertness of \([\text{Fe(TRA})P(OH)]^{4-}\) and \([\text{Ga(TRA})P(OH)]^{4-}\) are comparable. Interestingly, the \( K_{M(L)OH^{[OH]}} \) protonation constants indicate that the formation of \([\text{Fe(TRA})P(OH)]^{5-}\) intermediate takes place at significantly higher \(-\log[H^+]\) values than that of \([\text{Ga(TRA})P(OH)]^{5-}\) intermediate. However, the \( M(L)OH^{[OH]} \) rate constant of \([\text{Fe(TRA})P(OH)]^{5-}\) intermediate is about two orders of magnitude higher than that of \([\text{Ga(TRA})P(OH)]^{5-}\) intermediate, which indicates the considerably lower kinetic inertness of \([\text{Fe(TRA})P(OH)]^{5-}\) intermediate.

In order to compare the kinetic inertness directly, the half-lives (\( t_{1/2} = \ln(2)/k_d \)) of the dissociation reactions of \([\text{Fe(TRA})P]^{3-}\) and \([\text{Ga(TRA})P]^{3-}\) at \( p[H] = 7.4 \) have been calculated, utilizing the rate and equilibrium constants presented in Table 3. The \( t_{1/2} \) values of \( \text{Fe(TRA})P \) and \( \text{Ga(TRA})P \) are 1.1 \times 10^{5}, and 1.4 \times 10^{5} h, respectively, which indicates a similar kinetic inertness of \([\text{Fe(TRA})P]^{3-}\) and \([\text{Ga(TRA})P]^{3-}\) due to comparable \( M(L)OH^{[OH]} \) rate constants of the \([\text{Fe(TRA})P]^{4-}\) and \([\text{Ga(TRA})P]^{4-}\) complexes.
TABLE 3 | Rate constants characterizing the formation ($k_{\text{OH}}$) and dissociation ($k_{\text{d}}$) of [Fe(TRA P)$^{3-}$], [Ga(TRA P)$^{3-}$], [Ga(NOTA)], and [Ln(NOTA)] complexes (25°C).

| Formation kinetics | Dissociation kinetics |
|--------------------|----------------------|
| $k_{\text{OH}}$ /M$^{-1}$s$^{-1}$ | $k_{\text{OH}}$ /s$^{-1}$ | $k_{\text{d}}$ /s$^{-1}$ at pH = 7.4 | $t_{1/2}$/h at pH = 7.4 |
| [Fe(TRA P)$^{3-}$] | (3.37 ± 0.02) x 10$^4$ | (4 ± 1) x 10$^{-7}$ | 13.4 (1) | 1.8 x 10$^{-9}$ | 1.1 x 10$^5$ |
| [Ga(TRA P)$^{3-}$] | (1.47 ± 0.02) x 10$^5$ | (4.3 ± 0.5) x 10$^{-7}$ | 10.9 (1) | 1.4 x 10$^{-9}$ | 1.4 x 10$^5$ |
| [Ga(NOTA)$^a$] | 1.14 x 10$^5$ | – | – | – |
| [Ce(NOTA)$^b$] | 6.3 x 10$^7$ | – | – | – |
| [Ga(NOTA)$^a$] | 7.1 x 10$^7$ | – | – | – |
| [Er(NOTA)$^b$] | 5.5 x 10$^7$ | – | – | – |

$^a$Ref. (Morin and Toth, 2011); $^b$Ref. (Brucher and Sherry, 1993).

FIGURE 6 | Pseudo-first-order rate constants ($k_{\text{d}}$) of the ligand exchange reactions of [Ga(TRA P)$^{3-}$] (A,C) and [Fe(TRA P)$^{3-}$] (B,D) with H$_x$HBED ($x$) as a function of –log[H$^+$] and [OH$^-$] ($x$ = 0, 1, and 2). Solid lines and symbols represent calculated and experimental $k_{\text{d}}$ pseudo-first-order rate constants, respectively. ([Ga(TRA P)] = [Fe(TRA P)] = 0.2 mM, [H$_x$HBED] = 2.0 mM ($\bullet$), and 4.0 mM ($\Delta$); 0.15 M NaCl, 25°C).

SCHEME 3 | Proposed mechanism of the dissociation of [Fe(TRA P)$^{3-}$] and [Ga(TRA P)$^{3-}$] complexes ($x$ = 0 and 1).
and [Ga(TRAP)OH]^{4-} complexes. On the other hand, reliability of our kinetic model is supported by a good agreement of the dissociation half-life for [Ga(TRAP)]^{3-} at pH = 11 determined in this study (t_{1/2} = 86 h) with the literature value of t_{1/2} ≈ 60 h (Notni et al., 2010).

CONCLUSION

Due to the availability of ^{68}Ge/^{68}Ga generators, recent years have seen an ever-growing interest in the radionuclide ^{68}Ga^{III} for PET examinations. The corresponding radiopharmaceuticals generally contain ^{68}Ga^{III} in form of chelates, for which purpose dedicated bifunctional chelators are usually conjugated to biological targeting vectors. The carrier-free ^{68}Ga^{III} obtained by acidic elution from the generator may contain some metal ions as impurities in trace amounts. These metal ions, like Ti^{IV}, Fe^{III}, Cu^{II}, and Zn^{II}, may compete with the ^{68}Ga^{III} for the chelator’s binding sites. Hence, knowledge of the possible interactions of these ions and Ga^{III} with chelates are highly important.

In this work, the interaction of Ga^{III} and Fe^{III} ions with H_{4}TRAP, a phosphinic acid analog of H_{3}NOTA, was studied and compared. The stability constants of the [Ga(TRAP)]^{3-} and [Fe(TRAP)]^{3-} complexes were found to be very similar, as are their very low dissociation rates at physiological pH. The dissociation predominantly occurs via spontaneous dissociation of mono-hydroxo [M(TRAP)OH]^{4-} complexes and bis(hydroxo) [M(TRAP)(OH)_{2}]^{5-} intermediates. Similarly to the respective NOTA complexes, formation of Ga(TRAP) and Fe(TRAP) is slow and occurs by formation of the monoprotonated [M(HTRAP)]^{2-} intermediates. The stability of these intermediates is very high, presumably because both the phosphinate and carboxylate groups of the ligand are coordinated. However, although we observed an extraordinary similarity of the thermodynamic and kinetic properties of the Ga(TRAP) and Fe(TRAP) complexes, there is a small but important difference between the two systems: the formation rate of Ga(TRAP) is approximately three times higher than that of the Fe(TRAP), which has implications for the influence of Fe^{III} contaminations on ^{68}Ga labeling of TRAP.

Apparently, the previously observed selectivity of TRAP for ^{68}Ga^{III} over Fe^{III} is rooted in a totally different mechanism than the preference of TRAP for Ga^{III} over Cu^{II} and Zn^{II} (Simecek et al., 2013). Because Fe(TRAP) is formed more slowly than Ga(TRAP), formation of ^{68}Ga(TRAP) is preferred and even a 3-fold excess of Fe^{III} over TRAP does not substantially reduce the labeling yield. However, Fe(TRAP) is kinetically inert, and a higher excess of Fe^{III} ultimately inhibits the ^{68}Ga^{III} incorporation due to an irreversible consumption of all available TRAP. On the other hand, the TRAP complexes of Zn^{II} and Cu^{II} are formed much faster but they are not inert (Baranyai et al., 2015). Unlike Fe^{III}, TRAP-bound Cu^{II} and particularly Zn^{II} may therefore be readily displaced by Ga^{III} (Simecek et al., 2013), driven by a much higher thermodynamic stability of [Ga(TRAP)]^{3-} as compared to [Zn(TRAP)]^{4-} and [Cu(TRAP)]^{4+} (logK_{ML} of 26.24, 16.07, and 19.09, respectively) (Notni et al., 2010; Baranyai et al., 2015). Hence, in contrast to Fe^{III}, even high concentrations of Cu^{II} and particularly that of Zn^{II} do not completely inhibit ^{68}Ga labeling of TRAP, likewise resulting in a pronounced tolerance of these potential contaminants. We conclude that even a phenomenon of elementary character, namely, the selectivity of TRAP for Ga^{III} which manifests itself in a tolerance of remarkably high concentrations of different metal ion impurities during ^{68}Ga^{III} labeling, may rely on a variety of driving forces and molecular properties, thus requiring a detailed investigation of mechanistic details for thorough understanding.

AUTHOR CONTRIBUTIONS

AV and AF contributed to the equilibrium and kinetic characterizations; AW performed the ligand synthesis; EB, IT, AM, H-JW, JN, and ZB contributed to the evaluation of the physico-chemical parameters and to the manuscript preparation.

ACKNOWLEDGMENTS

Support by the Deutsche Forschungsgemeinschaft (grant #NO822/4-1 and SFB 824, project A10), the EU and the European Regional Development Fund (projects GINOP-2.3.2-15-2016-00008 and GINOP-2.3.3-15-2016-00004) are gratefully acknowledged. The project has been carried out in the frame of the EU COST Action CA15209: European Network on NMR Relaxometry.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fchem.2018.00170/full#supplementary-material

REFERENCES

Baes, C. F., and Mesmer, R. E. (1976). *The Hydrolysis of Cations*. New York, NY: London; Sydney; Toronto, ON: John Wiley & Son.

Baranyai, Z., Reich, D., Vagner, A., Weinseien, M., Toth, I., Wester, H. J., et al. (2015). A shortcut to high-affinity Ga-68 and Cu-64 radiopharmaceuticals: one-pot click chemistry trimerisation on the TRAP platform. *Dalton Trans.* 44, 11137–11146. doi: 10.1039/C5DT00576K

Bevilaqua, A., Gelb, R. L., Hebard, W. B., and Zompa, L. J. (1987). Equilibrium and thermodynamic study of the aqueous complexation of 1,4,7-triazacyclononane-N,N',N"-triacetic acid with protons, alkaline-earth metal cations, and copper(II). *Inorg. Chem.* 26, 2699–2706. doi: 10.1021/ic00263a029

Beyer, T., Townsend, D. W., Brun, T., Kinahan, P. E., Charron, M., Roddy, R., et al. (2000). A combined PET/CT scanner for clinical oncology. *J. Nucl. Med.* 41, 1369–1379.

Brucher, E., and Sherry, A. D. (1990). Kinetics of formation and dissociation of the 1,4,7-triazacyclononane-N,N',N"-triacetate complexes of cerium(III), gadolinium(III), and erbium(III) ions. *Inorg. Chem.* 29, 1555–1559. doi: 10.1021/ic00333a022
Clarke, E. T., and Martell, A. E. (1991). Stabilities of the Fe(III), Ga(III) and In(III) chelates of N,N,N′-triazacyclononanetricarboxylic acid. Inorg. Chim. Acta 181, 273–280. doi: 10.1016/S0020-1693(00)88621-8

Drahos, B., Kubicek, V., Bonnet, C. S., Hermann, P., Lukes, I., and Toth, E. (2011). TRAP, a powerful ligand set with versatile properties. Inorg. Chim. Acta 401, 1945–1951. doi:10.1016/j.inoche.2011.03.026

Farkas, E., Kosma, E., Petho, M., Herlihy, K. M., and Micera, G. (1998). Equilibrium studies on copper(II)- and iron(III)-monohydroxamates. Polyhedron 17, 3331–3342. doi:10.1016/S0277-5389(98)00113-2

Frank, R., and Patrick, J. R. (2010). The renaissance of the 68Ge/68Ga radionuclide generator initiates new developments in 68Ga radiopharmaceutical chemistry. Curr. Top. Med. Chem. 10, 1633–1668. doi:10.2174/156802610793176738

Gerald, C. F., C., Sherry, A. D., Marques, P. M. M., Alpoim, M. C., and Cortes, S. (1991). Protonation scheme for some triaza macrocycles studied by potentiometry and NMR spectroscopy. J. Chem. Soc. Perkin Trans. 2, 137–146. doi:10.1039/p2991000137

Irving, H. M., Miles, M. G., and Pettit, L. D. (1967). A study of some problems in determining the stoichiometric proton dissociation constants of complexes by potentiometric titrations using a glass electrode. Anal. Chim. Acta 38, 475–488. doi:10.1016/S0003-2670(01)80616-4

Levi, H. (1976). George von Hevesy memorial lecture. George Hevesy and his concept of radioactive indicators in retrospect. Eur. J. Nucl. Med. 1, 3–10. doi:10.1007/BF00253259

Ma, R., Motekaitis, R. J., and Martell, A. E. (1994). Stability of metal ion complexes of N,N-bis(2-hydroxybenzyl)ethylenediamine-N,N′-diacetic acid. Inorg. Chim. Acta 224, 151–155. doi:10.1016/0020-1693(94)04012-5

Mariko, T., and Susumu, T. (1977). The preparation of trivalent metal chelates with some N3O3-type ligands. Bull. Chem. Soc. Jpn. 50, 3413–3414. doi:10.1246/bcsj.50.3413

Morfin, J. F., and Toth, E. (2011). Kinetics of Ga(NOTA) formation from weak Ga-citrate complexes. Inorg. Chem. 50, 10371–10378. doi:10.1021/ic102445e

Notni, J. (2012). With Gallium-68 into a New Era? Nachr. Chem. 60, 465–469. doi:10.1515/nachrchem.2012.60.6.645

Notni, J., Hermann, P., Havlickova, J., Kotek, J., Kubicek, V., Plutnar, J., et al. (2010). A triazacyclononane-based bifunctional phosphinate ligand for the preparation of multimeric 68Ga tracers for positron emission tomography. Chem. Eur. J. 16, 7174–7185. doi:10.1002/chem.200903281

Notni, J., Simecek, J., Hermann, P., and Wester, H. J. (2011). TRAP, a powerful and versatile framework for gallium-68 radiopharmaceuticals. Chem. Eur. J. 17, 14718–14722. doi:10.1002/chem.201103503

Notni, J., Simecek, J., and Wester, H. J. (2014). Phosphinic acid functionalized polyazacycloalkane chelators for radiodiagnostics and radiotherapeutics: unique characteristics and applications. ChemMedChem. 9, 1107–1115. doi:10.1002/cmdc.201400055

Notni, J., and Wester, H.-J. (2018). Re-thinking the role of radioisotopes: towards a future concept for theranostic radiopharmaceuticals. J. Label. Compd. Radiopharm. 61, 141–153. doi:10.1002/jlcr.3582

Rösch, F. (2013). Past, present and future of 68Ge/68Ga generators. Appl. Rad. Isot. 76, 24–30. doi:10.1016/j.radisot.2013.05.010

Simecek, J., Hermann, P., Wester, H. J., and Notni, J. (2013). How is 68Ga labeling of macrocyclic chelators influenced by metal ion contaminants in 68Ge/68Ga generator eluates? ChemMedChem. 8, 95–103. doi:10.1002/cmdc.201200471

Simecek, J., Schulz, M., Notni, J., Plutnar, J., Kubicek, V., Havlickova, J., et al. (2012). Complexation of metal ions with TRAP (1,4,7-triazacyclononane phosphonic acid) ligands and 1,4,7-triazacyclononane-1,4,7-triacetic acid: phosphinate-containing ligands as unique chelators for trivalent gallium. Inorg. Chem. 51, 577–590. doi:10.1021/ic202103v

Simecek, J., Zemek, O., Hermann, P., Notni, J., and Wester, H. J. (2014). Tailored Gallium(III) chelator NOPO: synthesis, characterization, bioconjugation, and application in preclinical Ga-68-PET imaging. Mol. Pharm. 11, 3893–3903. doi:10.1021/mp400642s

Velikyan, I. (2011). Positron emitting [68Ga]Ga-based imaging agents: chemistry and diversity. Med. Chem. 7, 345–379. doi:10.2174/157340611796799195

Wadas, T. J., Wong, E. H., Weisman, G. R., and Anderson, C. J. (2010). Coordinating radiometals of copper, gallium, indium, yttrium, and zirconium for PET and SPECT imaging of disease. Chem. Rev. 110, 2885–2902. doi:10.1021/cr900325h

Zekany, L., and Nagypal, I. (1985). “PSEQUAD, ” in Computational Methods for the Determination of Formation Constants, ed D. Leggett (Springer), 291–353.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer, FG, and handling Editor declared their shared affiliation.

Copyright © 2018 Vágner, Forgács, Brücher, Tóth, Maiocchi, Wurzer, Wester, and Baranyai. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.