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Additives for the crystallization of proteins and nucleic acids

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Abstract

Numerous molecules have been described in literature as additives that were indispensable either for nucleation or growth of macromolecular crystals. In some cases, such additives were shown to improve the quality of the X-ray diffraction and to extend diffraction limits. We have investigated the effects of more than fifty compounds, belonging to several chemical families, on the crystallization of four model proteins (hen and turkey egg-white lysozymes, thaumatin, and aspartyl-tRNA synthetase from *Thermus thermophilus*). In addition, we have studied the crystallization of a ribonucleic acid from yeast, the transfer RNA specific for phenylalanine in the presence of synthetic polyamines. Crystals grown in the presence of the additives were optically evaluated and X-ray diffraction analyses were performed on selective crystals to compare their space group, cell parameters, and diffraction limit with those of controls. Whereas no changes in space group nor cell parameters were observed for the model proteins, significant improvements in diffraction limit were found when the transfer RNA was crystallized with certain synthetic polyamines.

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1. Introduction

Macromolecular crystallogenesis is a complex process dictated not only by physical–chemical parameters, like solubility, temperature, ionic strength, pH, pressure or viscosity, but also by biochemical properties such as particle purity and conformational homogeneity. In contrast to small molecules that are rigid and static, larger biological molecules are dynamic possessing flexible extensions and domains. Hence, conformational microheterogeneity imposes an entropic barrier for their crystal nucleation and growth [1]. Common methods for improving homogeneity involve elimination of mobile parts either by limited proteolysis

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[2,3] or by gene manipulation [4]. Another means to act on conformers is to perturbate their environment (i.e. their solvation shell, counterions, hydrogen bonding, etc.) with additives. Such compounds were useful for facilitating nucleation events [5], decreasing crystal solvent content [6], or preparing heavy atom derivatives [7].

In this study we have examined the effects of commercial and of novel small organic compounds on the crystallization of model macromolecules. Three commercial proteins, lysozymes from hen and turkey (HEWL, TEWL) and thaumatin, as well as a laboratory purified aspartyl-tRNA synthetase (AspRS) from *Thermus thermophilus* were crystallized in the presence of more than 50 additives. In addition, we have investigated the crystallization of yeast $tRNA^{Phe}$ in the presence of nine synthetic polyamines. Since tRNA is probably more sensitive to the presence of small ions than most proteins due to the anionic character of its external surface, we have examined the specific counterion effects imposed by polyamines of various sizes and charges for $tRNA^{Phe}$ crystal growth. We chose two methodological approaches. For proteins, conditions yielding large well-formed crystals diffracting at high resolution were used for the screening of compounds that could alter their crystallization. For RNAs, generally more difficult to crystallize, a nonoptimal crystallization condition of $tRNA^{Phe}$ was selected to detect improvements in crystal quality. Protein and RNA crystals were optically examined and some were subjected to preliminary crystallographic analyses to compare lattice parameters and diffraction limits.

2. Materials and methods

2.1. Chemicals, proteins and tRNA

Compounds added in protein crystallization assays are listed in Table 1. PEG 3000 and 6000 were recrystallized from acetone and diethylether [8]. Octyl glucoside was purified by column chromatography [9]. Ammonium sulfate was ultrapure (Aristar grade, BDH). [^{14}C] aspartate was from Amersham. Solutions were prepared with distilled sterile water (Fresenius, France) and filtered through Millex membranes of 0.2 μm pore size (Millipore). Their pH was adjusted to a value compatible with the crystallization conditions. Sodium azide was added as a bactericidal agent at 0.05% w/v (7.8 mM) in all protein solutions. Solutions of spermine, spermidine, erythritol, octyl glucoside and argininamide were stored in −20°C, the others at 4°C. Polyamines with chemical formulas displayed in Fig. 1 were synthesized as described: no. 1 [10], no. 2–3 [11,12], no. 4 [13], no. 5–6 and no. 8 [14], no. 7 [12] and no. 9 [14].

Ultrapure HEWL (6-times crystallized, dialyzed and lyophilized) was from Seikagaku Corp. (Tokyo, Japan, Cat. No. 100940 Lot E94Z05). TEWL (Cat. No. L-6255, Lot 64H7230), bovine serum albumin (Cat. No. 4503) and thaumatin (Cat. No. T-7638, Lot 108F0299) were from Sigma. AspRS from *T. thermophilus* was overproduced and purified as described [15]. $tRNA^{Phe}$ was purified from bulk yeast tRNA (Boehringer) by countercurrent distribution [16] followed by chromatography over BD-cellulose. Pure $tRNA^{Phe}$ was dialyzed against sterile water for two days and concentrated to 8 mg/ml with a Centricon-10 concentrator (Amicon).

Activity of AspRS in the presence of additives was assayed with bulk tRNA from yeast [17] after dilution in 100 mM HEPES pH 7.2 containing 1 mg/ml bovine serum albumin. Final aminoacylation medium contained 100 mM HEPES pH 7.2, 30 mM KCl, 10 mM $MgCl_2$, 2 mM ATP, 12 μg/μl $tRNA^{Asp}$, 50 μM [^{14}C] aspartate at 343 cpm/pmol, and additives (concentrations were 1/10 of stock solutions, Table 1). Assays were initiated by 2 μl AspRS solution at 110 μg/ml to 50 μl aminoacylation medium after 2 min incubation at 40°C. Samples of 15 μl were withdrawn after 3, 6 and 9 min and deposited on 1 cm^2 Whatmann 3MM paper treated in the conventional way [17]. Radioactivity was measured by liquid scintillation.

2.2. Crystallization experiments and crystallographic analysis

Crystals were grown in hanging drops on siliconized glass coverslips using the vapor diffusion technique in Linbro plates. Crystallization conditions are summarized in Table 2. Protein stock solutions were filtered on Ultrafree-MC membranes (Cat. No. UFC 30GV00, Millipore)

Table 1
Compounds added in protein crystallization assays

Chemical family	Chemical compounds (origin[a])		M_r	Stock solution	Concentration range
Carbohydrates, and derivatives	Ribose	(S - R7500)	150	1 M	1–200 mM
	Xylose	(M - 8689)	150	1 M	id.
	Fructose	(S - F0127)	180	1 M	id.
	Glucose	(R - 6780)	180	1 M	id.
	1-O-Methyl-α-D-Glucopyranoside	(S - M9376)	194	1 M	id.
	Saccharose	(M -7654)	342	1 M	id.
	Maltose	(S - M5885)	360	1 M	id.
	Trehalose	(S - T9531)	378	1 M	id.
	Stachyose	(S - S4001)	667	1 M	id.
Alcohols	Isopropanol	(CE - 415146)	60	1 M	id.
	Ethylene glycol	(M - 1.09621)	62	1 M	id.
	1,2-Propanediol	(S - P6209)	76	1 M	id.
	Glycerol	(F- 49780)	92	1 M	id.
	1,6-Hexanediol	(A - H1180-7)	118	1 M	id.
	2-Methyl-2,4-Pentanediol	(K -1828)	118	1 M	id.
	Erythritol	(S - E7500)	122	1 M	id.
	1,2,3-Heptanetriol	(S - H6011)	148	1 M	id.
	Adonitol	(S - A5502)	152	1 M	id.
	Arabitol	(S - A3381)	152	1 M	id.
	Xylitol	(S - X3375)	152	1 M	id.
	meso-Inositol	(M - 4720)	180	0.5 M	0.5–100 mM
	Mannitol	(S - M4125)	182	1 M	1–200 mM
	Sorbitol	(S - S1876)	182	1 M	id.
	PEG 400	(A - 20239-8)	400	30% v/v(~ 0.75 M)	0.03–6% v/v
	PEG 1000	(S - P3515)	1000	30% w/v(~ 0.3 M)	0.03–6% w/v
	PEG 3000	(M - 8.19015)	3000	30% w/v(~ 0.1 M)	id.
	PEG 6000	(A - 130192)	6000	30% w/v(~ 0.05 M)	id.
Amino acids and derivatives	Glycine	(M - 1.04201)	75	1 M	1–200 mM
	Alanine	(S - A7752)	89	1 M	id.
	Sarcosine	(S - S9881)	89	1 M	id.
	Proline	(S - P0380)	115	1 M	id.
	Taurine	(S - T0625)	125	0.5 M	0.5–100 mM
	Glycylglycine	(S - G1002)	132	1 M	1–200 mM
	Betaine	(S - B2752)	135	1 M	id.
	N,N-Dimethylglycine	(S - D6382)	140	1 M	id.
	Cysteine	(P - 23.255)	176	1 M	id.
	Glutamic acid	(M - 292)	184	1 M	id.
	Carnitine	(S - C9500)	198	1 M	id.
	Lysine	(P - 25050)	219	1 M	id.
	Argininamide	(S - A3913)	246	1 M	id.
Polyamines	Putrescine	(S - P7505)	161	1 M	id.
	Cadaverine	(S - C0500)	175	1 M	id.
	Spermidine	(S - S2501	254	1 M	id.
	Spermine	(S - S2876)	348	1 M	id.

Table 1 *Continued*

Chemical family	Chemical compounds (origin[a])		M_r	Stock solution	Concentration range
Surfactants	Non-detergent sulfobetaine 195	(C - 480001)*	195	1 M	id.
	Non-detergent sulfobetaine 201	(C - 480005)*	201	1 M	id.
	N,N-Dimethyldodecylamine-N-oxide	(S- D9775)	229	1 M	id.
	Octyl-β-D-Glucopyranoside	(C - 494459)	292	1 M	id.
	Hecameg**	(V)	335	1 M	id.
	Triton X-114	(S - X114)	537	1 M	id.
	CHAPS	(S - C3023)	615	0.5 M	0.5–100 mM
Other compounds	Dimethylsulfoxide	(R - 7029)	78	1 M	1–200 mM
	1,4-Dioxane	(M - 3118)	88	1 M	id.
	K thiocyanate	(M - 5125)	97	1 M	id.
	2,3-Butanedione monoxime	(S - B0753)	101	0.5 M	0.5–100 mM
	Trimethylamine-N-oxide	(S - T0514)	111	1 M	1–200 mM
	Polyvinyl Pyrrolidone	(A - 23, 425-7)	2.5×10^4	8×10^{-6} M	8×10^{-9}–1.6×10^{-7}M
	Polyethylene amine	(S - 33141)	3×10^4 –4×10^4	25% w/v ($\sim 7\times10^{-7}$ M)	0.025–5% w/v

Compounds are listed in order by increasing M_r in each chemical family. Concentrations of stock solutions and ranges explored in crystallization assays are indicated. [a]Manufacturers and catalog numbers are: A = Aldrich; Ap = Appligene; C = Calbiochem; CE = Carlo Erba; F = Flucka; K = Kodak; M = Merck; P = Prolabo; R = Roth; S = Sigma; Se = Serva; V = Vegatec. (*) Sulfobetaines were provided by Dr. L. Vuillard. (**) 6-O–(N-heptylcabamoyl)-methyl-α-D-glucopyranoside.

Table 2
Crystallization conditions and crystals of model macromolecules

Macromolecule Name, oligomeric structure and M_r	Crystallization condition Macromolecular concentration (*), precipitant, buffer, additives and temperature	Crystal Habit, space group and cell parameters
Hen egg white lysozyme Monomer, 14 300	40 mg/ml, 1 M NaCl 100 mM Na acetate pH 4.7, 20°C	Tetragonal prism, $P4_32_12$ $a = b = 79.2$ Å, $c = 38.0$ Å,
Turkey egg white lysozyme Monomer, 14 200	40 mg/ml, 1 M NaCl 100 mN Na acetate pH 4.7, 4°C	Hexagonal prism, $P6_122$ $a = b = 70.9$ Å, $c = 84.6$ Å
Thaumatin Monomer, 22 200	35 mg/ml, 0.7 M Na tartrate 100 mM ADA pH 6.5, 20°C	Tetragonal bipyramide, $P4_12_12$ $a, b = 58.6$ Å, $c = 151.8$ Å
Aspartyl-tRNA synthetase Homodimer, 132 000	7 mg/ml, 4.25 M Na formate, 25 mM Tris-HCl pH 7.5, 1 mM $MgCl_2$, 1 mM EDTA, 4°C	Orthorhombic prism, $P2_12_12_1$ $a = 61.4$ Å, $b = 156.1$ Å, $c = 177.3$ Å
tRNA^{Phe} Monomer, 25 000	8 mg/ml, 15%v/v MPD 100 mM Na cacodylate pH 6.5, 20 mM $MgCl_2$, 2 mM $CoCl_2$, 4°C	Hexagonal prism, $P6_222$ $a = b = 82$ Å, $c = 236$ Å

(*) Before mixing the macromolecular solution with the reservoir solution in a 1 : 1 volume ratio.

prior to crystallization. Compounds were added in reservoir solutions (1 ml for trials with lysozymes and 500 μl for thaumatin and AspRS) with 5, 20, 100 and 1000-fold dilution of additive stocks for HEWL crystallization and only 10-fold dilution of the stock for other proteins. Crystallization drops (final volumes of 5–20 μl) were prepared by mixing protein stocks with reservoir solutions containing additives in a 1 : 1 volume ratio. Prior to crystallization, tRNA samples in 100 mM Na cacodylate pH 6.5 and 20 mM $MgCl_2$ were heated to 60°C for 5 min and slowly cooled to 4°C. Drops were made by mixing 5 μl of tRNA solution and 5 μl reservoir solution (Table 2) containing varying concentrations of polyamines.

Protein crystals were mounted in Lindemann glass capillaries sealed with bee wax and irradiated with the Ni filtered Cuα radiation ($\lambda = 1.54$ Å) of a rotating anode generator operated at 50 kV and 100 mA. Diffraction data were collected on a Mac Science DIP 2000 imaging plate (Nonius). tRNA crystals were analyzed at 4°C with a Rigaku RU-200BH rotating anode X-ray generator equipped with a Huber graphite monochromator operating at 50 kV and 50 mA. Data were collected on an 30 cm diameter MAR Research IP detector. RNA crystals were evaluated with partial data sets (20–30% completness) in three sections of 15° (with 0.5° oscillations) in intervals of 45°. Maximum resolution data with $\langle I\rangle/\sigma\langle I\rangle \geqslant 3$ were scored for each tRNA crystal. Data were reduced using the HKL package [18].

3. Results

3.1. Choice of compounds and model macromolecules

A survey of soluble proteins listed in the biological macromolecule crystallization database shows that more than a hundred compounds of various chemical nature may positively influence nucleation and/or crystal growth [19]. Amongst those, some affecting positively macromolecular crystallization are presented in Table 3. They include sugars, alcohols, ions, polyamines and surfactants and improve molecular stability, nucleation, crystal growth and quality. In this study, the effects of 58 compounds (listed in Table 4) were analyzed. Further, the effects of nine synthetic polyamines (Fig. 1) on the crystallization of $tRNA^{Phe}$ were evaluated.

Model macromolecules were chosen on the basis of their availability and purity. Ultrapure HEWL was most abundant followed by TEWL and thaumatin. AspRS, a dimeric enzyme that charges the aspartic acid on $tRNA^{Asp}$, was a representative of large multidomain proteins most frequently encountered in research laboratories. Yeast $tRNA^{Phe}$ was used as a model for crystal growth studies of structured globular RNAs.

3.2. Effects on protein crystallization

Crystallization conditions (19) optimized for this study to produce a few large crystals (1–5 with a size of 0.8–1.5 mm) within one month in the absence of additives are summarized in Table 2 with the crystallographic parameters. We verified that these crystals diffract X-rays to a resolution limit of 1.8 Å for HEWL and of 2.0–2.5 Å for thaumatin, TEWL and AspRS. During the first trials with HEWL, cysteine and glutamic acid proved to be difficult to handle because of their low solubility and were not tested on other proteins (***n.a.*** in Table 4). In the presence of Hecameg, crystallization droplets containing the three other proteins detached from coverslips and the effect of this compound could not be determined (***n.d.*** in Table 4).

The effects of 56 compounds on the crystallization of the model proteins are summarized in Table 4. In brief, an effect was observed with 14 compounds on HEWL (at the two highest reagent concentrations), with seven compounds on TEWL, with four on thaumatin, and 17 on AspRS. The most tangible observations on the crystal growth of these proteins were variations in nucleation number, size, occurrences of twinned or cracked crystals and formation of urchin-like bundles of needles. Except in one case, there was no indication of protein denaturation (no precipitation) in drops that resulted from addition of the compounds. AspRS was chosen as a target to detect more discrete denaturation because changes in specific activity often reflect a structural perturbation.

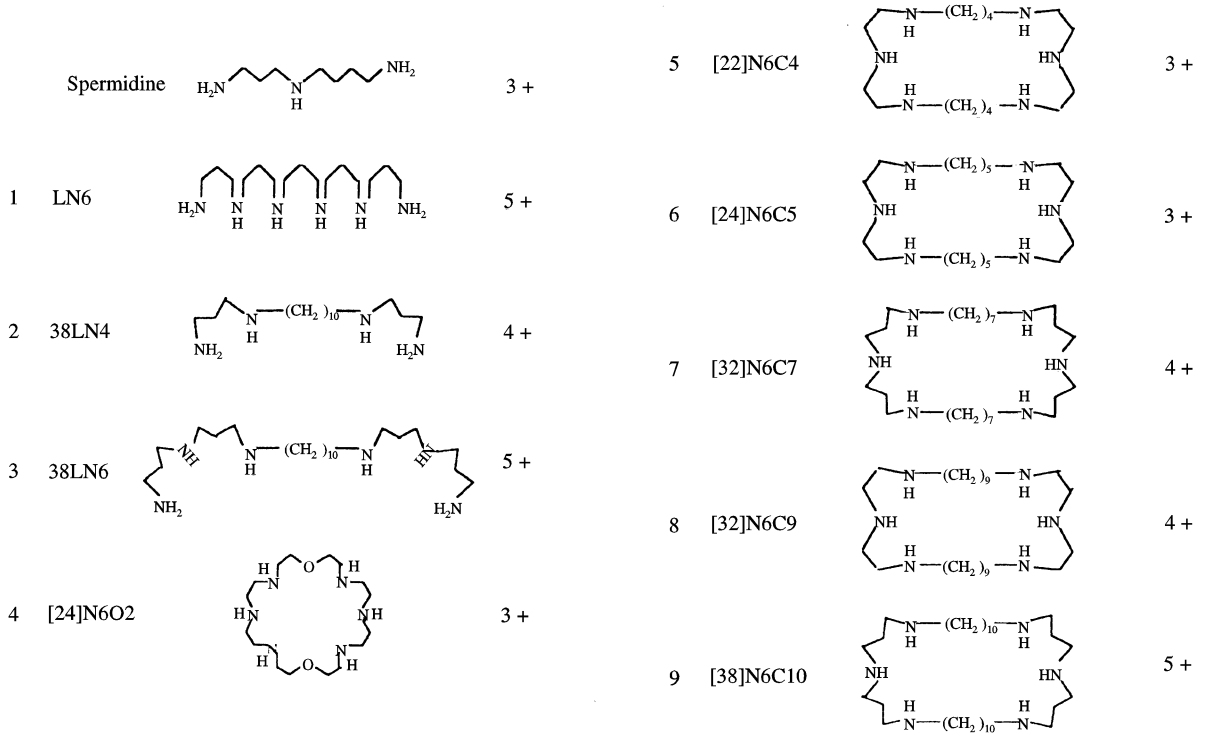


Fig. 1. Synthetic polyamines used to crystallize $tRNA^{Phe}$. The structures are shown with their corresponding identification numbers on the left and their protonation levels at pH 6.5 (on the right side). Spermidine is shown for comparison.

Table 3
Some compounds affecting positively macromolecular crystallization

Additives	Effects	Target macromolecules	Ref.
Sugars and alcohols	Increase of stability	T7 RNA pol	[22]
	Facilitation of nucleation	T7 RNA pol, fibronectin	[22,23]
	Cryoprotection		[24–26]
	Stabilization of the structure (glycerol)	$GlnRS/tRNA^{Gln}$	[19]
	Decrease of mosaic spread (glycerol)	T7 RNA pol	[1,27]
Dioxane	Decrease of growth kinetic	Hsc20	[28]
	Prevention of twinning	α-chymotrypsin	[29]
Cations	Increase of crystal lifetime in X-rays	His binding prot	[30]
	Changes in crystal habit (Ca^{2+},Mg^{2+})	Peroxidase	[31]
	Enhancement of resolution limit (Cd^{2+})	His, Leu-lle-Val binding prot	[30,32]
	Stabilization of the structure (Mg^{2+})	Nucleic acids	[33]
Polyamines	Decrease in charge repulsion, stabilization of the structure	Nucleic acids	[20]
	Enhancement of resolution limit	Glycogen debranching enzyme	[34]
Surfactants	Alteration of crystal habit, promotion of crystal growth (octyl glucoside)	Proteins, RNA, complexes	[35]
	Promotion of crystal growth (sulfobetaines)	HEWL	[5]

Table 4
Effects of compounds on protein crystallization and activity

Additives	Effects on number (*N*) and size (*S*) of crystals				Effect on activity AspRS
	HEWL	TEWL	Thaumatin	AspRS	
None	**1–2 large crystals**	**2–4 large crystals or urchins**	**3–5 large crystals**	**1–2 large crystals**	**1.0**
Ribose	ne	ne	ne	ne	1.3
Xylose	ne	ne	en	ne	ne
Fructose	**N**$^+$/**S**$^-$	ne	ne	ne	0.7
Glucose	ne	ne	ne	ne	ne
1-O-Methyl-Glucopyranoside	ne	ne	ne	ne	ne
Saccharose	ne	ne	ne	**N**$^+$	1.3
Maltose	ne	ne	ne	**N**$^{+\ +}$/**S**$^-$	**1.5**
Trehalose	**N**$^{+\ +}$/**S**$^-$	ne	**N**$^+$	**N**$^{+\ +}$/**S**$^-$	1.4
Stachyose	**N**$^+$/**S**$^-$	ne	ne	**N**$^+$/**S**$^-$	ne
Isopropanol	ne	ne	ne	ne	1.4
Ethylene glycol	**N**$^+$	ne	ne	**N**$^+$	**1.7**
1,2-Propanediol	ne	ne	ne	ne	0.8
Glycerol	ne	ne	ne	ne	ne
1,6-Hexanediol	ne	***s***	ne	ne	ne
2-Methyl-2,4-Pentanediol	ne	ne	ne	ne	ne
Erythritol	ne	ne	ne	ne	ne
1,2,3-Heptanetriol	ne	ne	ne	**N**$^+$/**S**$^-$	ne
Adonitol	**N**$^-$ or **s**	ne	ne	ne	ne
Arabitol	**N**$^-$ or **s**	ne	ne	ne	ne
Xylitol	ne	ne	ne	ne	ne
meso-Inositol [§]	ne	ne	ne	ne	ne
Mannitol	ne	ne	ne	**N**$^+$/**S**$^-$	ne
Sorbitol	ne	***t***	ne	ne	ne
PEG 400 [§]	ne	ne	ne	**N**$^+$/**S**$^-$	**2.5**
PEG 1000 [§]	**Longer**	***s***	ne	***p***	**0**
PEG 3000 [§]	ne	ne	ne	**N**$^+$/**S**$^-$	**1.7**
PEG 6000 [§]	**Longer**	ne	**N**$^+$/**S**$^-$	**N**$^+$/**S**$^-$	**2.5**
Glycine	ne	ne	ne	ne	ne
Alanine	ne	ne	ne	ne	0.7
Sarcosine	ne	ne	ne	ne	ne
Proline	ne	ne	ne	ne	**0.4**
Taurine	**Cracked**	***t***	ne	ne	ne
Glycylglycine	ne	ne	ne	ne	0.8
Betaine	ne	ne	***t***	ne	ne
N,N-Dimethylglycine	**N**$^{+\ +}$/**S**$^-$	ne	ne	ne	ne
Cysteine	ne	***n.a.***	***n.a.***	***n.a.***	ne
Glutamic acid	**N**$^+$/**cracked**	***n.a.***	***n.a.***	***n.a.***	ne
Carnitine	ne	ne	ne	ne	ne
Lysine	**N**$^+$/**cracked**	ne	ne	ne	**0.4**
Argininamide	ne	ne	ne	**N**$^{+\ +}$/**S**$^-$	**1.9**
Putrescine	ne	ne	ne	**N**$^+$/**S**$^-$	**2.5**
Cadaverine	ne	ne	ne	ne	ne
Spermidine	ne	ne	ne	ne	ne
Spermine	**N**$^+$/**cracked**	ne	ne	ne	0.5

Table 4 *Continued*

Additives	Effects on number (*N*) and size (*S*) of crystals				Effect on activity AspRS
	HEWL	TEWL	Thaumatin	AspRS	
Sulfobetaine NDSB195	ne	**s**	ne	ne	ne
Sulfobetaine NDSB201	ne	**s**	ne	**N$^+$/S$^-$**	1.4
N,N-Dimethyldodecyl amine-N-oxide	ne	ne	ne	ne	1.4
Octyl-β-D-Glucopyranoside	ne	ne	**N$^+$/S$^-$**	ne	**1.8**
Hecameg	ne	***n.d.***	***n.d.***	***n.d.***	**1.5**
Triton X-114	**Phase separation**	ne	ne	***s***	**2.0**
CHAPS [§]	ne	***s***	ne	ne	**1.6**
Dimethylsulfoxide	ne	ne	ne	**N$^+$**	1.3
1,4-Dioxane	ne	ne	ne	**N$^+$**	0.7
K thiocyanate	***u***	ne	ne	ne	ne
2,3-Butanedione monoxime [§]	ne	ne	ne	ne	**0.4**
Trimethylamine-N-oxide	**N$^+$**	ne	ne	ne	**1.8**
Polyvinyl Pyrrolidone [§]	ne	ne	ne	ne	**1.5**
Polyethylene amine [§]	ne	ne	ne	ne	**0**

Greater or smaller number of crystals are indicated by **N$^+$** or **N$^-$**. Likewise, crystal sizes that are bigger are indicated as **S$^+$**. Observation of crystallization trials are described as soluble protein (***s***), twinned crystals (***t***), urchin-like clusters of needles (***u***), precipitate (***p***), no effect (ne), not analyzed (***n.a.***) and not determined (***n.d.***). Final additive concentrations used were 100 mM in the equilibrating reservoir solution unless otherwise indicated ([§], see Table 1). In the case of HEWL, 1, 10, 50 and 200 mM of final concentration of additives were prepared in the reservoir except where noted ([§]) and effects indicated are for the two highest additive concentrations. Enzymatic activity of AspRS is expressed as the ratio of initial rate of tRNA aminoacylation in the presence of additive (10-fold dilution of stock solution) to initial rate in the absence of additive. Ratios having more than 20% change of activity to that of the controls are indicated. Differences in enzymatic activity exceeding 40% are shown in bold characters.

Table 4 lists the activities measured in the presence of each compound relative to that in its absence. Several compounds resulted in an increase superior to 50%. On the other hand, seven compounds caused a 20–60% decrease in activity. The most prominent effect was the abolishment of activity with polyethylene amine (that obviously traps tRNA) and PEG 1000 (that was contaminated by various ions detected by conductivity measurements, as described by Jurnak [36]).

The shape of crystals obtained in the presence of most compounds did not differ from that of controls. Tetragonal crystals of HEWL grown with PEG 1000 and 6000 were elongated along their *c*-axis but X-ray analyses indicated that their cell parameters fluctuated by no more than $\pm 0.5\%$ (as did control crystals) and that their diffraction limit was not altered. AspRS crystals grown in the pres ence of 100 mM glycerol were also analyzed to recognize any effects on unit cell parameters. Our interest in the role of glycerol as a stabilizing agent for glutaminyl-tRNA synthetase-tRNA^{Gln} crystals from *E. coli* [20] as well as its usage in crystal soaking for cryogenic data collection drew our attention to this. Cell parameters and diffraction limits for the glycerol grown AspRS crystals were about the same as those of controls.

3.3. *Effects on tRNA^{Phe} crystallization*

Yeast tRNA^{Phe} can be crystallized in 12 different crystal forms under various conditions reviewed by Dock-Bregeon et al. [21]. Crystallization conditions that produced the hexagonal habit using MPD as the principle crystallizing agent in the presence of spermidine were chosen because this form was the fastest and easiest to obtain. Since these hexagonal crystals are generally of poor quality having a

diffraction limit of no more than 8 Å, this particular crystallization condition was very suitable to detect a discrete enhancement of crystal quality.

Synthetic polyamines differ from natural polyamines (spermine, spermidine, putrescine, cadaverine, and thermin) in shape, spread and number of charges: three polyamines (no. 1–3) are linear and five compounds are macrocycles (no. 4–9) (Fig. 1). Compounds 1 and 2 are linear hexamines with two dipropylenetriamine units connected by linear trimethylene and decamethylene chains. Compound 3 is the tetramine analog of compound 2. Compounds 4, 5, 6, and 8 contain diethylenetriamine units whereas all other contain dipropylenetriamine groups. Various crystallization conditions were explored with synthetic polyamines and all trials produced crystals. Only the most dramatic results are described here.

In most cases, increasing carbon lengths as well as overall charges appear to promote the growth of single and larger crystals compared to those grown with the traditional polyamine, spermidine (Table 5). The most striking cases are shown in Fig. 2 where hexagonal crystals grown in the presence of polyamines 4 (panel B), 6 (panel C) and 8 (panel D) are compared to $tRNA^{Phe}$ crystals obtained with spermidine (panel A) under the same conditions. In each case, a given concentration of synthetic polyamine produced crystals that were greater in volume and less numerous than those grown with the same concentration of spermidine. Generally, increasing polyamine concentrations decreased clustering while increasing crystal size (not shown).

Results of X-ray analyses used to evaluate effects of polyamines on the quality of $tRNA^{Phe}$ crystals are summarized in Table 5. The diffraction limit varies with the polyamine added. Crystals grown in the presence of polyamines 1 and 2 displayed marked improvements in their resolution even though the crystal sizes were similar to that reached with spermidine. $tRNA^{Phe}$ crystals grown with polyamines 4–9 were larger with less nucleation and diffracted to as much as 2.2 Å higher in resolution than crystals grown with spermidine. The diethylenetriamine unit polyamine compounds 4, 6 and 8 seem to have the most positive influence on $tRNA^{Phe}$ as evaluated by size and diffraction limit (Table 5). On the other hand, polyamine 3 did not give rise to any changes. In fact, the crystal size was generally smaller having the same resolution as crystals obtained with spermidine.

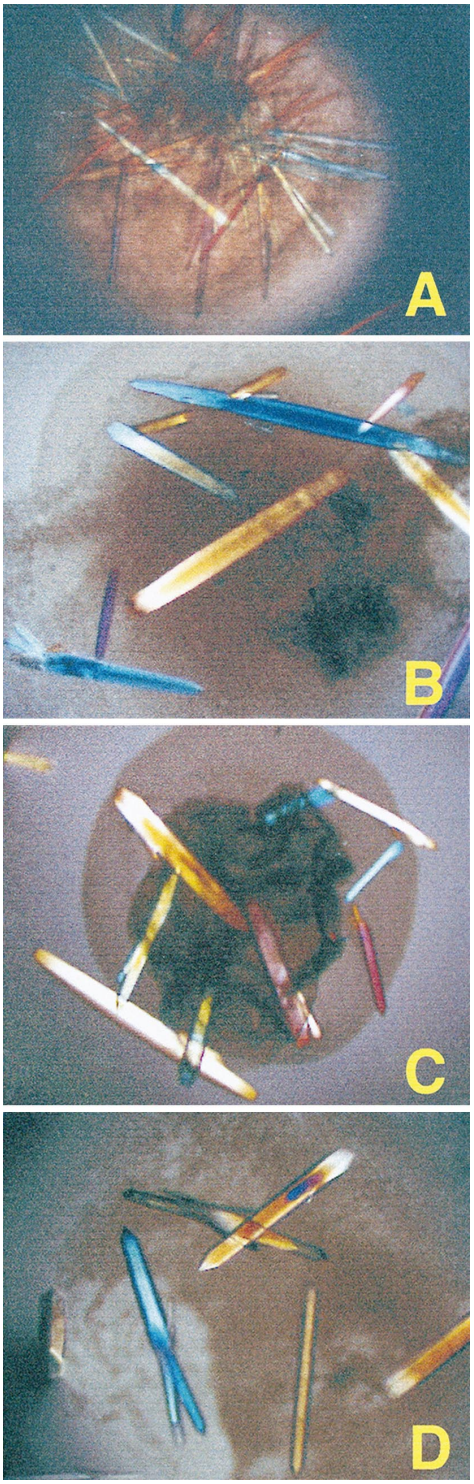
4. Discussion and conclusion

4.1. Additives for protein crystallization

Altogether many compounds added in crystallization assays were without any detectable effect. As to the three model proteins with the most stable structures (see Table 4), amongst carbohydrates, the diose trehalose strongly enhanced nucleation of HEWL and thaumatin, probably as a consequence of an osmotic effect. Short-chain alcohols and polyols had moderate effects, either on nucleation or crystal habit. Amino acids and their derivatives

Table 5
Crystal of $tRNA^{Phe}$ grown in the presence of synthetic polyamines

Polyamines	Crystal size (mm^3)	Maximum resolution (Å)	Overall $\langle I\rangle/\sigma\langle I\rangle$	Overall R_{merge} (%)
Spermidine	$0.7\times0.1\times0.1$	8.0	11.5	6.2
1	$0.8\times0.2\times0.2$	5.0	13.5	12.3
2	$0.5\times0.1\times0.1$	6.0	11.5	14.3
3	$0.3\times0.05\times0.05$	8.0	9.4	11.5
4	$1.2\times0.3\times0.3$	3.5	10.5	5.2
5	$0.5\times0.2\times0.2$	6.0	9.4	10.5
6	$1.0\times0.3\times0.3$	4.0	11.5	7.3
7	$0.8\times0.3\times0.3$	6.0	11.3	8.4
8	$0.6\times0.3\times0.3$	3.5	12.2	6.7
9	$0.6\times0.2\times0.2$	5.0	10.5	8.3



acted mainly on nucleation or led to crystal cracking as did spermine on HEWL. Surfactants did not affect very much the crystallization when compared to controls. Other compounds had only a minor action on nucleation.

AspRS was most sensitive to the addition of various chemicals. About one-third of the compounds examined affected its crystallization by influencing either nucleation, crystal size or solubility. More than 50% of the compounds interfered with its activity, 20 compounds enhanced it. The latter results are explained by structural modifications that result in more homogenous population of enzymatically competent molecules. Amongst the 17 compounds affecting the crystallization of AspRS, 14 (i.e. 80%) have an effect on its activity and 12 of them enhance it. This shows the importance of activity assays to verify the biological relevancy of protein structures in crystallization media. In the case of AspRS, the highest activity was measured with PEGs, probably as a consequence of their effectiveness, at low ionic strength, to promote a higher affinity in ligand binding. X-ray diffraction analyses have shown that crystals grown from PEG are more isotropic than those obtained in control experiments while retaining the same unit cell symmetry and dimensions (data not shown).

4.2. Additives for nucleic acid crystallization

Few RNAs have been crystallized to date and only a limited number of 3D structures have been solved by X-ray crystallography (reviewed by Masquida et al. [37]) because most crystals do not diffract beyond 4–5 Å resolution. RNAs are more susceptible to the effects of small cations than are most proteins. Polyamines, such as spermine or spermidine, act as specific counterions to the negative phosphate groups of the RNA backbone and their concentration ratio with metal cations, such as Mg^{2+}, are substantial factors towards obtaining

Fig. 2. Crystal habits of $tRNA^{Phe}$ crystals influenced by polyamine additives. Hexagonal $tRNA^{Phe}$ crystals grown in the presence of spermidine (panel A), polyamines 4 (panel B), 6 (panel C) and 8 (panel D).

high-quality crystals [38,39]. In general, polyamines are important in protein synthesis and in particular for tRNA folding. Both rate and precision of tRNA aminoacylation are dependent on polyamine concentration [40]. The nine synthetic polyamines used here stimulate the in vitro transcription of single- and double-stranded DNA templates at levels dependent on their size, shape, protonation degree and concentration [41]. This study gives evidence that they influence positively the structure and packing of tRNAs and improve their crystallization.

High-quality tRNA crystals for X-ray studies are obtained only when crystallization is performed in the presence of spermine [42,43] which was found in the crystals at a precisely defined locus. The bound polyamine molecule was further shown to distort the tRNA backbone in a different way than counterions [43]. Synthetic polyamines influence crystal growth most likely in a similar manner by electrostatic interactions with polyanion charges. In the case of spermidine with three positive charges, a high free energy electrostatic interaction should be created. However, the hexagonal tRNA crystal form reported in this study is obtained at low ionic conditions representing perhaps an interaction of only two of the three possible positive charges on spermidine with the backbone phosphate groups on the tRNA.

The best tRNA crystals were those obtained with macrocyclic compound number 4. Its compact structure and three positive charges are probably very favorable in maximizing the interactions with the RNA. This may stabilize or engender conformity by specific interaction with the tRNA giving rise to a more ordered crystalline lattice. Similarly, other cyclic polyamines also may have favorable electrostatic interactions with the RNA with varying degrees of specific binding or group interactions which ultimately improve crystal stability for crystallization. Compounds 1 and 2 are linear polyamines stabilizing tRNA molecule probably by accessing the major groove at one end of the anticodon stem or the variable region as observed for spermine [38]. Moreover, strong binding to the RNA can exist from a higher number of positive charges resulting in a greater free energy of electrostatic interactions. Compound 3 is among the longest of the synthesized linear polyamines. It does not seem to have influence on any improvement of tRNA crystal growth. Even though there is a high number of positive charges, the length of the linear chain may exceed the size of the binding domain on the tRNA molecule. This investigation does not preclude the possibility that synthetic polyamines may affect the binding of Mg^{2+} ions on RNA as it was reported for spermine [42]. Such studies on the interaction of Mg^{2+} ion and the synthetic polyamines on tRNA crystallization remains to be done.

4.3. Conclusion and prospective

This investigation supports that compounds of various chemical nature may influence the crystallization of a protein or a nucleic acid. Indeed, in the case where no satisfying crystals can be prepared, the refinement of crystallization conditions should include a systematic screen of nondenaturing additives. Such compounds should not be used only in millimolar concentrations but also in the range of 50–200 mM since most do not affect crystal quality at such concentrations. Thus for cryocrystallography, proteins can be readily crystallized in the presence of cryoprotectants since they prevent the formation of ice [24–26].

Compounds playing the role of counterions to RNA molecules are important in stabilizing their globular structure. As shown here, synthetic polyamines with different sizes and charges have a wide range of influence on tRNA crystal size, nucleation number and X-ray diffraction limit. These molecules will find wide applications in the crystallization of other RNAs, especially those of large molecular weights.

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References

[1] R. Sousa, Methods Enzymol. 276 (1997) 131.
[2] J.-P. Waller, J.-L. Risler, C. Monteilhet, C. Zelwer, FEBS Lett. 16 (1971) 186.
[3] F. Jurnak, A. Rich, D. Miller, J. Mol. Biol. 115 (1977) 103.
[4] J.A. Barwell, A. Bochkarev, R.A. Pfuetzner, H. Tong, D.S. Yang, L. Frappier, A.M. Edwards, J. Biol. Chem. 270 (1995) 1088.
[5] L. Vuillard, T. Rabilloud, R. Leberman, C. Berthet-Colominas, S. Cusack, FEBS Lett. 353 (1994) 294.
[6] B. Schick, F. Jurnak, Acta Crystallogr. D 50 (1994) 565.
[7] C.W.Jr. Carter, R.M. Sweet, (Eds.), Methods Enzymol. 276 Part A (1997) 1.
[8] A. Ducruix, R. Giegé (Eds.) Crystallization of Nucleic Acids and Proteins: A Practical Approach, IRL Press, Oxford, 1992, new edition in preparation.
[9] B. Lorber, J.B. Bishop, L.J. DeLucas, Biochim. Biophys. Acta 1023 (1990) 254.
[10] B. Dietrich, M.W. Hosseini, J.-M. Lehn, R.B. Sessions, Helvetica Chim. Acta 66 (1983) 1262.
[11] C. Oriol-Audit, M.W. Hosseini, J.-M. Lehn, European J. Biochem. 151 (1985) 557.
[12] M.W. Hosseini, J.-M. Lehn, Helvetica Chim. Acta 69 (1986) 587.
[13] M.B. Comarmond, P. Plumere, J.-M. Lehn, Y. Agnus, R. Louis, R. Weiss, O. Kahn, I. Morgenstern-Badaran, J. Am. Chem. Soc. 104 (1982) 7074.
[14] M.W. Hosseini, J.-M. Lehn, J. Am. Chem. Soc. 109 (1987) 7047.
[15] J.D. Ng, B. Lorber, J. Witz, A. Théobald-Dietrich, D. Kern, R. Giegé, J. Crystal Growth 168 (1996) 50.
[16] G. Dirheimer, J.-P. Ebel, Bull. Soc. Chim. Biol. 49 (1967) 1679.
[17] H.D. Becker, R. Giegé, D. Kern, Biochemistry 35 (1996) 7447.
[18] Z. Otwinowsky, W. Minor, Methods Enzymol. 276 (1997) 307.
[19] G.L. Gilliland, M. Tung, D.M. Blakeslee, J. Ladner, Acta Crystallogr. D 50 (1994) 408.
[20] M.A. Rould, J.J. Perona, T.A. Steitz, Nature 352 (1991) 213.
[21] A.-C. Dock-Bregeon, B. Lorber, D. Moras, G. Pixa, J.-C. Thierry, R. Giegé, Biochimie 66 (1984) 179.
[22] D. Jeruzalmi, T.A. Steitz, J. Mol. Biol. 274 (1997) 748.
[23] I. Pechnik, J. Nachman, K. Ingham, G.L. Gilliland, Proteins: Struc. Fonc. Genet. 16 (1993) 43.
[24] G.A. Petsko, J. Mol. Biol. 96 (1975) 381.
[25] D.W. Rodgers, Methods Enzymol. 276 (1997) 183.
[26] E.F. Garman, T.R. Schneider, J. Appl. Crystallogr. 30 (1997) 211.
[27] E.P. Mitchell, E.F. Garman, J. Appl. Crystallogr. 27 (1994) 1069.
[28] J.R. Cupp-Vickery, L.E. Vickery, Protein Sci. 6 (1997) 2028.
[29] D.M. Blow, Trends Biochem. Sci. 22 (1997) 405.
[30] S. Trakhanov, F.A. Quiocho, Protein Sci. 4 (1995) 1914.
[31] J.F.W. Petersen, J.W. Tams, J. Vind, H.D. Svensson, H. Dalboge, K.G. Welinder, S. Larsen, J. Mol. Biol. 232 (1993) 989.
[32] S. Trakhanov, D.I. Kreimer, S. Parkin, G. Ferro-Luzzi Ames, B. Rupp, Protein Sci. 7 (1998) 600.
[33] A.-C. Dock, D. Moras, in: A. Ducruix, R. Giegé (Eds.), Crystallization of Nucleics Acids and Proteins: A Practical Approach, IRL Press, Oxford, 1992, p. 145.
[34] P.M.D. Fitzgerald, N.B. Madsen, J. Crystal Growth 76 (1986) 600.
[35] A. McPherson, S. Koszelak, H. Axelrod, J. Day, L. Robinson, M. McGrath, R. Williams, D.J. Cascio, J. Crystal Growth 76 (1986) 547.
[36] F. Jurnak, J. Crystal Growth 76 (1986) 577.
[37] B. Masquida, E. Westhof, in: S. Neidle (Ed.), Oxford Handbook of Nucleic Acid Structure, Oxford University Press, Oxford, 1998, in press.
[38] G.J. Quigley, M.M. Teeter, A. Rich, Proc. Nat. Acad. Sci. USA 75 (1978) 64.
[39] M.E. McMahon, V.A. Erdmann, Biochemistry 21 (1982) 5280.
[40] R.B. Lotftfield, E.A. Eigner, A. Pastuszyn, J. Biol. Chem. 256 (1981) 6729.
[41] M. Frugier, C. Florentz, W. Hosseini, J.-M. Lehn, R. Giegé, Nucleic Acid Res. 22 (1994) 2784.
[42] J.A. Ladner, J.T. Finch, A. Klug, B.F.C. Clark, J. Mol. Biol. 72 (1972) 99.
[43] M.M. Teeter, G.J. Quigley, A. Rich, in: T.G. Spiro (Ed.), Nucleic Acid–Metal Ion Interactions, ch. 4, Wiley-Interscience, New York, 1980.