Application of the effects of ionic strength reducing agents in the purification and crystallization of chitinase A

Yannis Papanikolau* and Kyriacos Petratos

Institute of Molecular Biology and Biotechnology (IMBB)-FORTH, PO Box 1527, 71110 Heraklion, Greece. E-mail: papaniko@imbb.forth.gr

The effects of ionic strength reducing agents may find a large number of applications. Based on these effects, we have redesigned the purification scheme of Chitinase A (ChiA) from *Serratia marcescen*. This scheme led to reproducibly crystallizable enzyme in both salting-in and salting-out conditions, which are presented here. Herein, we demonstrate some experimental applications of the ionic strength reducing agents theory and, in parallel, provide further evidence of the theory's correctness. Finally, we report a new crystal form produced recently in salting-in crystallization experiments. This form may allow the co-crystallization of ChiA mutants with longer substrates.

Keywords: protein purification; crystallization; interactions; solubility; ionic strength reducing agents

1. Introduction

Ionic strength reducing agents (Papanikolau & Kokkinidis, 1997) are in general organic compounds that reduce the ionic strength of aqueous salt solutions. These compounds favor the formation of salt clusters, with at least 2 types of ions per cluster. This idea is based on the Bjerrum electrolytic dissociation theory (Bockris & Reddy, 1970; Padova, 1972; Vaslow, 1972). Ionic strength reducing agents may have strong effects in chromatographic methods, precipitation and thus crystallization itself. The idea of the ionic strength reducing agents unifies all crystallization experiments in aqueous solutions and, at the same time subdivides the precipitation events into two main areas: the generalized salting-in and salting-out area. According to this, organic solvents and polyethylene glycol (PEG) induce salting-in effects, as they decrease the concentration of free ions in the solution. Ionic interactions dominate in the salting-in area. Apart from that, ionic strength reducing agents lessen salting-out effects. Polar as well as hydrophobic interactions dominate in the salting-out area.

Chitinase A (ChiA) is one of the chitinolytic enzymes from *Serratia marcescens.* It belongs to the family 18 of glycosyl-hydrolases superfamily (Henrissat & Bairoch, 1996). The precursor protein consists of 563 residues, of which the first 23 residues constitute the leader peptide that is removed upon secretion (Watanabe *et al.*, 1997) and therefore the mature protein consists of 540 amino acids with M_r 58515 Da. The measured isoelectric point of ChiA is 5.8. This is in agreement to the calculated value. The crystal structure of ChiA has been originally determined (Perrakis *et al.*, 1994) at 2.3 Å resolution. Biochemical and kinetic studies of ChiA and ChiB have been carried out by Brurberg *et al.* (1996). It was recently shown (Uchiyama *et al.*, 2001) that ChiA is an exo-chitinase cleaving $(NAG)_2$ (di-N-acetyl-glucosamine) in a processive manner from the reducing-end of crystalline chitin. The latter finding agrees with the results of our structural studies of ChiA mutants co-crystallized with substrates (Papanikolau *et al.*, 2001). In order to investigate in higher detail the structure-function relationship for ChiA from *S. marcescens*, we have focused on the improvement of the quality of the protein crystals.

2. Materials and methods

2.1. Conductivity data elucidate the effect of ionic strength reducers

In table 1, we measure conductivity in mixed aqueous electrolyte solutions, with a Jencons PCM3 conductivity meter. These conductivity data and the respective standard deviations were obtained from three independent experiments. These measurements clearly indicate that, the higher the amount of methanol, PEG 200, PEG 6000 and glycerol in the solution the lower the conductivity and thus the ionic strength measured. Substances like methanol, PEG 200, PEG 6000 and glycerol, that reduce the ionic strength of their solution are named ionic strength reducing agents or for simplicity reducers.

Table 1
Increasing concentration of methanol, PEG 200, PEG 6000 and glycerol decreases the conductivity and thus the ionic strength of the solutions

a: 20mM sodium acetate buffer pH 5.5, 100 mM NaCl	
Methanol in % v/v	Conductivity in mS
0	11.1 ± 0.2
10	9.8 ± 0.2
20	8.0 ± 0.2
30	7.0 ± 0.3
40	6.4 ± 0.3

b: 20mM sodium acetate buffer pH 5.5, 2 M NaCl	
PEG 200 in % v/v	Conductivity in mS
0	115.4 ± 0.7
10	90.5 ± 0.7
20	69.1 ± 0.7
30	48.5 ± 0.2
40	33.1 ± 0.2

c: 50mM sodium citrate buffer pH5.0, 100 mM ammonium sulfate	
PEG 6000 in % w/v	Conductivity in mS
0	24.0 ± 0.1
10	17.2 ± 0.1
20	12.1 ± 0.1

d: 50mM tris hydrochloride buffer pH 8.0, 1.5 M ammonium sulfate	
Glycerol in % v/v	Conductivity in mS
0	139.2 ± 0.7
10	106.8 ± 0.7
20	80.4 ± 0.7
30	55 ± 1
40	35 ± 1

2.2. Protein expression and previous purification method

The ChiA protein is overexpressed by thermo-induction in the *Escherichia coli* strain A5745. The purification of ChiA was originally carried out according to the method described previously (Vorgias *et al.*, 1992). According to this method, the secreted protein was isolated from the growth medium by ammonium sulfate precipitation to 80 % saturation. The precipitated proteins were dissolved in buffer A (20 mM Tris (pH 8.0), 1 mM EDTA, 0.1 phenylmethyl sulfonylfluoride and 1 M ammonium sulfate) and directly applied to a Phenyl sepharose CL-6B column equilibrated in the same buffer. The bound chitinase was eluted at the very end of the descending ammonium sulfate gradient from 1 to 0 M and during extensive washing with 20 mM Tris (pH 8.0). The protein was about 75 % pure as judged by SDS-PAGE. The enzyme was further purified with a Q-Sepharose column equilibrated with 20 mM Tris (pH 8.0). The protein was isolated in the flow through nearly homogeneous.

2.3. New purification method

In order to obtain crystals that diffract to high resolution, we have introduced a novel purification protocol. The culture medium with the secreted protein was loaded on a Sepharose CL-6B with 1.5 M ammonium sulfate in 20 mM Tris (pH 7.5) and washed with 1.2 M ammonium sulfate in 20 mM Tris (pH 7.5). An ammonium sulfate gradient (1.2 – 0 M) was applied. The fractions corresponding from 0.7 to 0.2 M ammonium sulfate, containing the ChiA, were pooled

 © 2002 International Union of Crystallography • Printed in Denmark – all rights reserved

and dialyzed against 20 mM sodium acetate (pH 4.8). PEG (polyethylene glycol) 6000 was added to the protein solution to 15 % (w/v) final concentration. The resulting protein solution was applied to SP-Sepharose cation exchanger. A linear gradient of increasing NaCl concentration (0 - 0.2 M) and decreasing PEG 6000 concentration (15 – 0 % (w/v)) was applied. The ChiA containing fractions, from 10.5 % (w/v) PEG 6000 and 0.06 M NaCl to 6 % (w/v) PEG 6000 and 0.12 M NaCl, were pooled and diluted 3 times with nanopure water. This protein solution was applied on a Hydroxyapatite column, washed with 0.5 mM NaCl unbuffered and eluted with 200 mM ammonium sulfate also unbuffered solutions.

2.4. Crystallizations

2.4.1. Ionic strength reducers in salting-out crystallization experiments. As reported earlier (Vorgias *et al.*, 1992), ChiA was originally crystallized with ammonium sulfate as the precipitating agent. In an attempt to improve the 'salting-out' crystallization conditions, we employed the acquired knowledge (Papanikolau & Kokkinidis, 1997) on the ionic strength reducing agents. We screened ammonium sulfate as the precipitating agent at different pH values. The screenings were done in hanging-drop vapor diffusion setups. Ammonium sulfate at 1 M precipitates ChiA at pH values of 7 to 8, giving crystalline precipitate. A number of ionic strength reducers (or simply reducers) were screened as inhibitors of the salting-out effect of ammonium sulfate, e.g. methanol, ethanol, iso-propanol, PEG 200, glycerol, PEG 400 and PEG 6000. In Figures 1a and 1b, ammonium sulfate was the precipitating agent in 'salting-out' experiments. In Figure 1a, PEG 200 was the ionic strength reducer. In Figure 1b, glycerol was the ionic strength reducer. The higher the concentration of ammonium sulfate, the higher the concentration of PEG 200 or glycerol required to solubilize the protein. All drops were formed by mixing 1 µl of protein stock solution and 4 µl of reservoir solution. The buffer in all drops was 100 mM Heppes pH 7.2. At 1.7 M ammonium sulfate and 10% (v/v) PEG 200, needle-like crystals were observed. Glycerol was found to be not as good reducer as PEG 200.

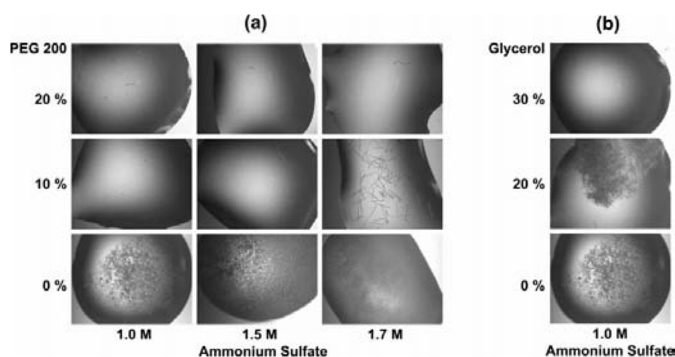

Figure 1
The effects of ionic strength reducing agents in salting-out experiments with ammonium sulfate as the precipitating agent in hanging-drop vapor diffusion crystallization experiments of ChiA. **(a)** On the horizontal axis from left to right, ammonium sulfate concentration is increased as indicated. On the vertical axis from bottom to top, PEG 200 concentration in % (v/v) is increased as indicated. PEG 200 is the ionic strength reducer. **(b)** Ammonium sulfate concentration is 1 M. From bottom to top, glycerol concentration in % (v/v) is increased as indicated. Glycerol is the ionic strength reducer.

In addition to ammonium sulfate, we also screened for salting-out crystallization conditions with other salts, namely NaCl, KCl, sodium phosphate and sodium citrate. These precipitating agents were screened at a variety of concentrations versus different buffers and pH values. Sodium citrate was proved to give the best results. Sodium citrate was also screened against a variety of ionic strength reducers such as methanol, ethanol, iso-propanol, glycerol, PEG 200, PEG 400 and PEG 6000. In Figure 2, sodium citrate at pH 7.2 was the precipitating agent in 'salting-out' experiments and PEG 200 was the ionic strength reducer. The higher the concentration of sodium citrate, the higher the concentration of PEG 200 required to solubilize the protein. All drops were formed by mixing 1 µl of protein stock solution and 4 µl of reservoir solution. Crystalline precipitate was observed. The best results for the salting-out crystallization experiments were obtained with 6 µl hanging-drops made of 4 µl protein solution and 2 µl of reservoir solution (0.75 M Na-citrate buffer pH 7.2 and 20% (v/v) methanol). Rod shaped crystals suitable for diffraction measurements (smallest dimension 0.3 mm) appeared within 3-4 days at 18 ^{o}C, which belong to space group $C222_1$, with one protein molecule per asymmetric unit. They diffract to at least 1.5 Å resolution. The crystal packing remains essentially the same as it was for the crystals obtained earlier (Perrakis *et al.*, 1994).

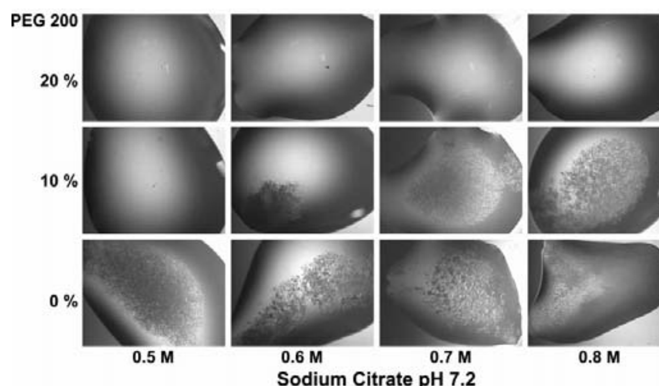

Figure 2
The effects of ionic strength reducing agents in salting-out experiments with sodium citrate as the precipitating agent in hanging-drop vapor diffusion crystallization experiments. From left to right, the concentration of sodium citrate pH 7.2 is increased as indicated. From bottom to top, PEG 200 concentration in % (v/v) is increased as indicated. PEG 200 is the ionic strength reducer.

2.4.2. Ionic strength reducers in salting-in crystallization experiments. Various ionic strength reducing agents, e.g. methanol, ethanol, iso-propanol, MPD, PEG 200, PEG 400, PEG 4000 and PEG 6000 were used as precipitating agents in our salting-in crystallization experiments. These precipitating agents were screened at a variety of concentrations versus different buffers and pH values. Crystal-like needles could be seen in experiments with 40 % (v/v) methanol or ethanol and 30 % (v/v) iso-propanol. The best-formed crystals were produced from 20-25 % (w/v) PEG 4000 or PEG 6000 at pH values from 4.8 to 8.5. The ionic strength reducing effect of PEG 6000 was regulated by the addition of different salts like NaCl, KCl, sodium citrate, sodium acetate and ammonium sulfate. In Figures 3a and 3b, PEG 6000 precipitates ChiA due to 'salting-in' effect. The higher the concentration of PEG 6000, the higher the concentration of salt required to solubilize the protein. In Figure 3a, the salt used was NaCl and in Figure 3b, ammonium sulfate. In both Figures, all drops were formed by mixing 1 µl of protein stock solution and 4 µl of reservoir solution. The buffer in all reservoir solutions was 50 mM sodium acetate pH 5.5.

The structure of ChiA was originally solved from crystals obtained under 'salting-in' crystallization conditions (Perrakis *et al.*, 1994). Hanging drops of protein solution mixed 1:1 with reservoir solution were equilibrated against 1 ml of reservoir solution (20-22 % PEG 4000, 0.15-0.2 M ammonium sulfate, 0.5-1 % (v/v) iso-propanol and 0.1 M acetate buffer, pH 5.2). The precipitating agent was PEG 4000 and iso-propanol. Ammonium sulfate and the acetate buffer regularize the salting-in effect. The crystals produced from PEG 4000 were not as rigid and sturdy as the crystals obtained from sodium citrate. This was noticed upon crystal-mounting and diffraction data collection.

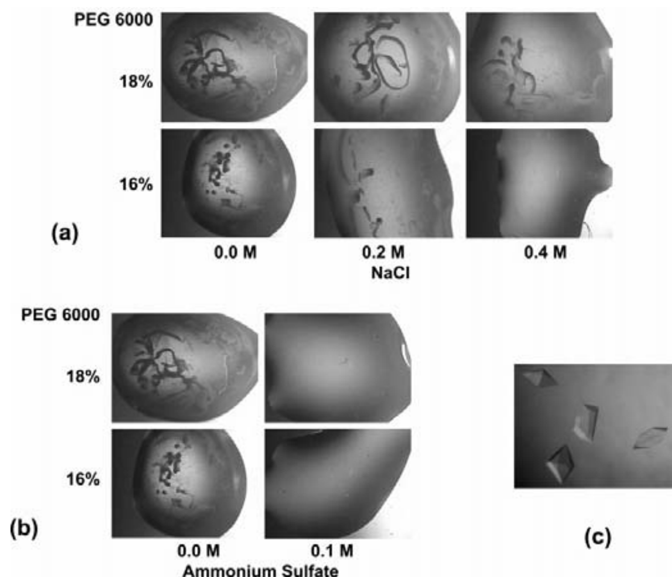

Figure 3
The effects of salts in salting-in experiments with PEG 6000 as the precipitating agent in hanging-drop vapor diffusion crystallization experiments. PEG 6000 is the ionic strength reducer in these experiments. **(a)** From bottom to top, PEG 6000 concentration in % (w/v) is increased as indicated. From left to right, NaCl concentration is increased as indicated. NaCl decreases the precipitating effect of PEG 6000. **(b)** From bottom to top, PEG 6000 concentration in % (w/v) is increased as indicated. From left to right, ammonium sulfate concentration is increased as indicated. Ammonium sulfate decreases the precipitating effect of PEG 6000. **(c)** Crystals that belong to space group $P2_12_12_1$.

2.4.3 Cryoprotectants in salting-in crystallization experiments. Cryo-data collection is lately the method of choice in protein crystallography. The selection of the proper solution for cryo-protection is a major problem for the acquisition of the best diffraction data from a given crystal. In order to produce crystals in a cryo-protection solution, we screened salting-in conditions with PEG 6000 in the presence of glycerol or PEG 200. At low concentrations (up to 15 % (v/v)), these additives did not change dramatically the crystallization results.

A new crystalline form (Fig. 3c) was obtained with 20 % PEG 6000 and 30–40 % (v/v) PEG 200 as precipitating agents. 50 mM ammonium sulfate and 50 mM sodium citrate buffer at pH 5.2 were the regulators of the ionic strength reducing effect of PEG 6000 and PEG 200. The new crystals belong to space group $P2_12_12_1$ with unit cell parameters: a= 87.3 Å, b= 113.0 Å and c= 116.5 Å. They are very rigid in manipulations and diffract to at least 1.7 Å resolution. This crystal form contains 2 protein molecules in the asymmetric unit.

3. Results

The original purification scheme of the enzyme (Vorgias *et al.*, 1992) though fast, did not consistently yield crystallizable protein. In addition, the protein samples were precipitating at concentrations higher than 10 mg/ml. This could not improve by pH or ionic strength adjustments. These observations along with SDS-PAGE experiments indicated lack of homogeneity and, for this reason, we redesigned the purification scheme.

In the original purification scheme the protein was eluted from the Phenyl sepharose matrix after extensive washing with buffer solution. This indicates that the protein is tightly bound to the matrix, suggesting that a less hydrophobic matrix is required for better results. Therefore, we replaced Phenyl sepharose by Sepharose CL-6B in our new purification scheme. This substitution resulted in elution of the enzyme inside the decreasing ammonium sulfate gradient. The substitution of Phenyl sepharose by Sepharose CL-6B had also an economic impact. Phenyl sepharose was suffering from irreversible clogging. As a result, it had to be replaced by fresh material after every 2 or 3 applications. In contrast, Sepharose CL-6B can be regenerated easily and used several times.

Although ChiA is homogeneous from the first step of Sepharose CL-6B, as judged by SDS gel, it still precipitates at concentrations higher than 10 mg/ml in a variety of pH and ionic strength values. Generally, it is recommended that a number of different properties of a protein are employed in a purification scheme. Thus, it is a common practice in our laboratory to screen for at least three different chromatographic matrices. We also prefer to screen crystallization conditions with protein concentrations greater than 20 mg/ml. Consequently, we screened for a second purification step.

The second matrix utilized in the original purification was an anion exchanger (Q Sepharose), which was used in a negative mode. ChiA in this step passes in the flow through, while some contaminations are absorbed on the matrix. Generally, the negative use of a matrix is not recommended.

ChiA binds neither to Q Sepharose anion exchanger at pH higher than its isoelectric point nor to SP Sepharose cation exchanger at pH lower than its isoelectric point, as is expected from the theory of ion exchange chromatography.

According to our experiments, the positively charged ChiA does not bind to SP Sepharose at 20 mM sodium acetate buffer pH 4.8. We also know from crystallization experiments that ChiA is precipitating at 20 mM sodium acetate buffer pH 4.9 in the presence of 20 % (w/v) PEG 6000, which is an ionic strength reducer. In the presence of PEG 6000 the electrostatic interactions are reinforced. This led to the finding that ChiA binds to SP Sepharose at 20 mM sodium acetate buffer at pH 4.9 in the presence of 20 % (w/v) PEG 6000. Therefore, SP Sepharose was selected as a second matrix in the purification scheme of ChiA. The elution from the matrix was obtained by decreasing the ionic strength reducer PEG 6000. For even better results, we also increased the salt concentration in the gradient. Thus, we applied a mixed gradient of increasing NaCl and decreasing PEG 6000.

The third and final step in our new purification scheme utilizes Hydroxyapatite chromatography. This step is necessary in order to remove PEG 6000 from the protein solution.

The protein produced by the new purification scheme could be concentrated easily up to 50 mg/ml. We believe that ChiA binds non-specifically polysaccharides, which are found in the growth medium. A number of ChiA molecules could bind to a long polysaccharide chain giving rise to a less soluble oligomeric cluster. These sugar impurities are removed during our purification process. As a result, the solubility of the pure protein increases as reported. Furthermore, the crystallizability of the enzyme becomes instantaneous, i.e. ChiA crystallizes under a wide variety of

conditions within hours. The pure enzyme was stored in 2 mM Tris-HCl buffer at pH 7.5, at a final concentration of 30 mg/ml. For long-term storage, the protein stock solution was kept at -20 °C.

4. Discussion

The purification scheme that we originally applied in our studies of ChiA was inadequate to reproducibly yield crystallizable protein. In order to overcome this situation, we created a completely new purification scheme for the enzyme. The pure protein obtained from the new scheme is readily crystallizable under a variety of conditions.

In the electrolyte aqueous solutions, there are three types of interactions: the long-range electrostatic interactions, the sort-range polar interactions (μ-stacking included) and the van der Waals (hydrophobic) interactions. The ionic strength reducers, by decreasing the dielectric constant of the medium, increase the strength of the electrostatic interactions. In addition, they reduce the dissociation of electrolytes (Bjerrum) and eventually the ionic strength of the solution. Therefore, in the presence of ionic strength reducers, e.g. PEG 6000, a protein binds stronger on an ion exchange matrix (e.g. S Sepharose). This effect is proportional to the concentration of the ionic strength reducer. Electrostatic interactions prevail in salting-in conditions. The fact that increased concentration of salt enhances protein solubility both in the absence and presence of ionic strength reducers, indicates salting-in effect. Ionic strength reducers act as precipitating agents in the salting-in area

All ions and molecules of an aqueous solution participate in the hydration shell of the protein. At high concentrations, molecules and individual ions compete for the formation of this hydration sphere. Salting-out is the condition under which a molecule or an ion cannot form the necessary hydration sphere in order to stay in solution. In this case, the molecules and ions interact with each other. This applies to both precipitation and hydrophobic interaction chromatography (HIC). Polar interactions dominate in salting-out. Thus, the interacting molecules in salting-out conditions are mainly connected *via* polar interactions. Therefore, Sepharose CL-6B is a polar interaction chromatographic matrix (PIC). The conflict between hydrophobic interaction chromatography (HIC) and reverse phase chromatography (RPC) does not really exist (Principles and Methods, Hydrophobic Interaction Chromatography Manual, Pharmacia, ISBN 91-970490-4-2). There is only one hydrophobic interaction chromatography namely the reverse phase chromatography. The pure hydrophobic nature of molecules is not exposed in aqueous solutions. In the salting-out area salts are the precipitating agents.

ChiA is an excellent protein model for examining the effects of ionic strength reducing agents. It crystallizes both by salting-in and salting-out in the presence of a variety of precipitating agents in a wide range of concentrations and pH values. In addition, it can be purified to homogeneity in large quantities (about 100 mg per 5 lt culture). We hope that the newly obtained crystal form will allow the co-crystallization of ChiA mutants with even longer substrates, e.g. $(NAG)_{10}$.

The generalized salting-in and salting-out effects affect all biological macromolecules that can be dissolved in aqueous electrolytic solutions. The salting-in area of such a macromolecule should be carefully screened. Stock solutions of macromolecules should have the lowest possible ionic strength. This means, that the concentration of salt or buffer in the stock solution should be less than 1 mM, if possible. If a macromolecule is insoluble under such conditions, then the minimal amount of salt should be used for salting-in crystallization. In both salting-in and salting-out areas many different precipitating agents should be tested. Finally, it should be noted that, the higher the purity of the protein and the reagents used in crystallization experiments, the better and more reproducible the obtained results.

The above simplistic description of what happens in mixed protein aqueous electrolyte solutions, satisfies to a great extent our experimental data. An exact detailed analysis of such solutions is highly complicated. It is known that parameters, like the dielectric constant used in our description, are very complex functions. The ionic strength reducers may participate in the formation of the hydration cosphere of ions or even macromolecules, making the description of the solute even more complicated. More experimental data, like the conductivity measurements we presented here, are needed in order to describe the nature of the mixed aqueous electrolyte solutions.

References

Bockris, J. & Reddy, A. K. N. (1970). Editors. *Modern Electrochemistry*, Vol. 1. New York: Plenum Press.

Brurberg, M. B., Nes, I. F. & Eijsink, V. G. H. (1996). *Microbiology*, **142**, 1581-1589.

Henrissat, B. & Bairoch, A. (1996). *Biochem. J.* **316**, 695-696.

Padova, J. (1972). *Water and Aqueous Solutions: Structure, Thermodynamics and Transport Processes*, edited by R. A. Horne, pp. 109-174. New York: Wiley.

Papanikolau, Y. & Kokkinidis, M. (1997). *Protein Eng.* **10**(8), 847-850.

Papanikolau, Y., Prag, G., Tavlas, G., Vorgias, C.E., Oppenheim, A.B. & Petratos, K. (2001). *Biochemistry*, **40**, 11338-11343.

Perrakis, A., Tews, I., Dauter, Z., Oppenheim, A. B., Chet, I., Wilson, K. S. & Vorgias, C. E. (1994). *Structure*, **2**, 1169-1180.

Uchiyama, T., Katouno, F., Nikaidou, N., Nonaka, T., Sugiyama, J. & Watanabe, T. (2001). *J. Biol. Chem.* **276**, 41343-41349.

Vaslow, F. (1972). *Water and Aqueous Solutions: Structure, Thermodynamics and Transport Processes*, edited by R. A. Horne, pp. 465-518. New York: Wiley.

Vorgias, C. E, Kingswell, A. J. & Dauter, Z. (1992). *J. Mol. Biol.* **226**, 897-898.

Watanabe, T., Kimura, K., Sumiya, T., Nikaidou, N., Suzuki, K., Suzuki., M., Taiyoji, M., Ferrer, S. & Regue, M. (1997). *J. Bacteriol.* **179**, 7111-7117.

 Received 23 March 2002 • Accepted 7 August 2002