CHAPTER 3

Effect of Salt on Activity and on the Structure-Function Related Behavior of NG-27 Xylanase
INTRODUCTION

In the previous chapter, unfolding of the NG-27 xylanase was studied thoroughly with respect to temperature and chaotropic agents. It was found that unfolding of the xylanase occurs extraordinarily slowly and that the NG-27 xylanase unfolds through a partially-structured intermediate state displaying enzymic activity. This chapter describes the results of experiments designed to explore the effect of salts like NaCl, KCl and KI on the activity and the structure function related behavior of the NG-27 xylanase.

Salts can alter the structure, stability, solubility and/or activity of an enzyme through specific binding interactions, or through bulk effects exerted at the level of the enzyme’s solvent environment [von Hippel & Schleich 1969, Arakawa & Timasheff, 1985, Arakawa, et. al., 1990, Pace, C.N. 1990, Arakawa & Timasheff, 1991, Kita, et. al., 1994, Lin & Bolen, 1995, Baldwin, R.L., 1996]. It has been known for many years now that enzymes from halophilic organisms [e.g., microbes living in the multi-molar KCl-containing waters of the dead sea] undergo inactivation, dissociation and occasionally structural unfolding when concentrations of salts are lowered down to sub-molar levels [Eisenberg & Wachtel, 1987, Zaccai & Eisenberg, 1990, Eisenberg, 1995]. Similarly, enzymes from non-halophilic organisms too have been observed to be either unfolded, inactivated, or precipitated by the presence of high (multimolar) concentrations of salts [Eisenberg & Wachtel, 1987, Zaccai & Eisenberg, 1990]. This indicates that enzymes tend to retain integrity of their structure-function only under conditions supporting the growth and activity of the host organisms and not in both types of salt environment.

The effect of a salt on an enzyme depends, of course, on both the structural-biochemical characteristics (and evolutionary background) of the enzyme, and on the nature of the salt itself [Arakawa, et. al., 1990, Baldwin, R.L., 1996, Sinanoglu & Abdulnur, 1965, Melander & Horvath, 1977, Timasheff & Arakawa, 1989, Lin, & Timasheff, 1996, Bonnette, et. al., 1994, Jensen, et. al., 1995]. ‘Salting-out’ has a stabilizing effect on protein structure; this effect is mediated through an increase in the surface tension of water, which results in the preferential exclusion of salt (co-solvent) molecules from the hydration shell surrounding the protein. The reverse effect of structural destabilization is seen with salts that are good at mediating ‘salting-in’. While such interpretations suffice to explain the conformational responses of many proteins, the anomalous behavior of a halophilic homolog of malate dehydrogenase [Pundak &
Eisenberg, 1981a,b, Zaccai, et. al., 1989] indicates that a simple interpretation involving ligand exclusion from the protein’s surface may not always present the true picture regarding effects of salts on protein structure and stability. Further, effects on activity are likely to be even harder to generalize than effects on structure: (a) because the microstructural characteristics of active sites have been known for long to be capable of being independent of the overall stabilities of protein three-dimensional structures, since active sites often display a higher conformational flexibility and labiality to denaturing influences [Tsou, C.L., 1993], and (b) because the effects of salts on enzyme activity are likely to depend to a significant extent on the chemical nature of the reaction catalyzed, and not merely on the retention of the microstructure of active site residues.

Results described in this chapter demonstrate that the NG-27 xylanase remains structured, as well as functional, both in low-salt environments and in multi-molar salt solutions. Most intriguingly, there was a progressive enhancement of activity with increase in salt concentration. Even more intriguing was the observation that the enzyme appeared to be equivalently stabilized by both KCl and KI, even though KCl is expected to effect salting-out of the protein, whereas KI is expected to effect salting-in, and exert a chaotropic effect on the three-dimensional structure [Timasheff & Arakawa, 1989]. Neither salt actually caused any detectable loss of solubility at the protein concentration used in experiments reported here (0.05 mg/ml). In summary, the NG-27 xylanase remained structurally stable and functional under conditions supporting the growth and activity of both halophilic and non-halophilic microbes. With a mild inclination for multi-molar salt solutions, it displayed marked stability to both salting-in and salting-out salts.
Results and Discussion

3.1. Facultative tetramerization through predominantly electrostatic interactions.

Preliminary studies with unpurified material from culture broth have indicated the enzyme to be (i) stable and functional at alkaline pH, with optimal activity at pH 8.4, and (ii) optimally active at a temperature of around 70 °C (Gupta et al., 1992, Gupta et al., 2000). At the alkaline pH of 8.4, the NG-27 xylanase was a tetramer under conditions of low ionic strength, using gel filtration chromatography on Superdex-200 columns on a Pharmacia SMART system and appropriate molecular weight markers [chapter 2 Fig-2.3]. Lowering of pH was observed to effect a facile dissociation to a monomeric state with increase in elution volume. pH of half dissociation was pH ≈ 6.8 (Figure 3.1a). The enzyme was originally purified and chromatographed in 50 mM Tris buffer (pH 8.4) without any salt. Raising of ionic strength through inclusion of moderate concentrations of salts such as NaCl or KCl also resulted in facile dissociation of the enzyme, with half dissociation at ≈125 mM KCl (Figure 3.1b). These happen, however, without any accompanying changes in structure as could be monitored by fluorescence emission spectroscopic measurements. Generally buffers used for purification of proteins often contain salts such as NaCl or KCl at concentrations of 100 to 250 mM (at which the NG-27 xylanase would be substantially, or wholly dissociated). The associated or dissociated enzyme could be considered to be native, depending on one’s perspective, since no structural changes appeared to accompany dissociation-reassociation.

The dissociation-association equilibrium was judged to be attained readily, through a rapid interconversion of monomeric and tetrameric forms, since (a) gel filtration chromatograms obtained in experiments conducted immediately following addition of KCl are identical to profiles obtained through extended incubations of protein with salt. Furthermore, (b) at all concentrations of KCl between 0 and 500 mM, the xylanase was observed to migrate on the column as a single species with an elution volume intermediate to that of the monomeric and tetrameric forms, instead of as two separate species eluting at 1.38 ml and 1.66 ml respectively (Fig.3.1b). It may be noted that two separate elutions, accompanied by a two-state inversion of populations as a function of increasing salt, would have indicated that interconversions between the
Figure 3.1a

Change in volume of elution of the NG-27 xylanase from a Superdex-200 SMART column occurring as a function of change in pH, showing dissociation effected by lowering of pH.
Figure 3.1b

Change in volume of elution of the xylanase from the same column as a function of change in the concentration of the salt, KCl, showing dissociation effected by increase in ionic strength. Plotted alongside are values for fractional change in $\lambda_{\text{max}}$, showing that there is no effect of dissociation on the fluorescence characteristics and, therefore, on the structure of the enzyme. The tetrameric form of the NG-27 xylanase shows an elution volume $\approx 1.38$ ml, while the dissociated form shows an elution volume $\approx 1.66$ ml.
monomeric and tetrameric forms tend to occur on a time scale that is much larger than that of a standard chromatographic experiment (30 minutes). If this were so, molecules loaded on the column as monomers would migrate throughout the experiment as monomers, and molecules loaded as tetramers would migrate as tetramers, yielding separate elutions. This was not observed; evidently, therefore intermediate elution volumes owe to time- and population-averaging of the migration of the enzyme, and indicate rapid interconversions.

Notably, there was no detectable CD spectroscopic or spectrofluorimetric alteration in the characteristics of the enzyme upon incubation with salt, as a consequence of either the presence of multimolar concentrations of KCl, or of the dissociation effected by KCl; this establishes that there was no significant conformational alteration effected by the salt.

3.2. Slow unfolding in presence of salt (unfolding of monomer)

Effect of electrolyte-induced dissociation: Dissociation of the NG-27 xylanase was complete by about 300 mM KCl. When the xylanase was heated in the presence of 500 mM KCl [Figure 3.2], the salt had two effects on thermal unfolding. The rate of unfolding was higher than that in the absence of KCl, and unfolding could not proceed to completion; the wavelength of maximal emission of the protein shifted only as far as 348.5 nm. [In the absence of KCl the protein emits maximally at 352-353 nm]. It appeared that salt stabilizes some region of the protein’s structure. The stabilizing effect was more dramatic in the presence of 2M KCl. Under these conditions, the protein could be seen to have been stabilized to such an extent that the rate of melting of structure was slowed down relative to that observed in absence of KCl.

3.3. KCl effects structural stabilization (altering thermodynamics/kinetics of unfolding)

The monomeric form of the xylanase in 2M KCl was found to have altered stability to unfolding by thermal means. The fractional unfolding effected through ten minutes of incubation at each of a set of predetermined temperatures, both in the presence of 2 M KCl and in the absence of KCl [through monitoring of changes in the wavelength of maximal fluorescence emission of the enzyme] was therefore assessed. This
Figure 3.2

Thermal unfolding of xylanase monitored spectrofluorimetrically as a function of temperature and time of incubation in the presence of 500 mM KCl.
experiment showed that, at all temperatures, the NG-27 xylanase population unfolded to a lesser degree in the presence of 2 M KCl (Fig. 3.3a), with a higher temperature of half-unfolding. It is to be noted that the temperature of half-unfolding is not referred as the $T_m$, because equilibrium was not reached in 10 minutes of incubation; indeed, as remarked earlier, equilibrium is reached only after about two hours of incubation. It thus appears that in the presence of 2M KCl since unfolding fails to proceed to completion, a kernel of structure within the overall three dimensional structure of the monomeric form of the enzyme is stabilized. The emission wavelength maximum of the enzyme (containing 11 tryptophan residues distributed over its entire structure) shifted from 334 nm to 346 nm, and not all the way up to 352 nm. In other words, the effective fractional unfolding was only $\approx 0.7$, even with temperatures of incubation as high as 84.5 °C (Fig. 3.3a), or with longer durations of incubation. The stabilization of structure is also apparent from experiments monitoring the rate of unfolding effected through incubation at a high temperature. For example, it is clearly seen [Fig-3.3b] that upon incubation at a temperature of 71.5 °C, the enzyme unfolds more slowly, as a monomer, in the presence of 2 M KCl, than as a tetramer in the absence of KCl. Collectively, these observations indicate that multimolar concentrations of KCl stabilize the structure of the NG-27 xylanase in a manner that both slow down unfolding and keeps unfolding from progressing to completion.

3.4. Effect of salt on GdnCl and urea mediated unfolding.

To analyze further the differences in the unfolding effected by GdnCl and urea, and to examine in greater detail the lack of complete unfolding by urea, the enzyme was incubated with urea in the presence of an electrolyte [in 250 mM and 2 M NaCl]. At the lower concentrations, the enzyme dissociated significantly in either NaCl or KCl (Figure 3.1b), while at higher concentration complete dissociation was ensured. One reason for performing this experiment was to see if exposing the enzyme to urea under dissociating conditions would release the kinetic barrier to unfolding. At the lower concentration of 250 mM NaCl (Figure 3.4a), exposure of tryptophan residues occurred somewhat faster than in the absence of the electrolyte (Figure 3.4b). A greater extent of exposure was effected by a lower concentration of urea at all time points. The release of the kinetic barrier to unfolding by urea however was not observed; and unfolding still took nearly a
Presence of 2M KCl causes the population of NG-27 xylanase to unfold to a lesser extent than in the absence of KCl, as assessed spectrofluorimetrically following 10 minute incubations at various temperatures. Notably, unfolding in the presence of 2M KCl does not go to completion.

Figure 3.3a

*Incomplete unfolding in presence of salt.* Presence of 2M KCl causes the population of NG-27 xylanase to unfold to a lesser extent than in the absence of KCl, as assessed spectrofluorimetrically following 10 minute incubations at various temperatures. Notably, unfolding in the presence of 2M KCl does not go to completion.
Figure 3.3b

Altered kinetics of unfolding in presence of salt. The slow unfolding of the tetrameric form of the NG-27 xylanase (without KCl) is slowed down even further in the monomeric form (in 2M KCl). Assessment of unfolding was carried spectrfluorimetrically.
Figure 3.4a

Spectrofluorimetric measurements of changes effected in the $\lambda_{\text{max}}$ of the NG-27 xylanase as a function of urea concentration and days of incubation, in the presence of 250 mM NaCl. The figure shows that the lower concentration of salt hastens unfolding in the presence of urea.
Figure 3.4b

Spectrofluorimetric measurements of changes effected in the $\lambda_{\text{max}}$ of the NG-27 xylanase as a function of urea concentration and days of incubation, showing the extremely slow kinetics of unfolding.
week to reach equilibrium. Intriguingly, the enzyme appeared to have been stabilized considerably by the higher concentration of NaCl, since no unfolding could be detected after 7 days of incubation in the presence of 6M urea and 2 M NaCl. (Figure 3.4c).

3.5. **Gel filtration chromatographic investigation of the effect of dissociating concentrations of NaCl**

The results displayed in Figure 3.5 reveal some interesting facets of the enzyme’s behavior, and further confirm our conclusions regarding kinetic stabilization of three-dimensional structure. At pH 8.4, the enzyme normally elutes from an analytical SMART Superdex-200 column at ≈1.38 ml; corresponding to the molecular weight of the tetrameric form of the NG-27 xylanase. Addition of NaCl to a concentration of 250 mM caused the protein to elute as a species of much smaller hydrodynamic volume due to dissociation, at just over 1.6 ml, whereas addition of urea to a concentration of 5 M independently caused the protein to elute as a species of larger hydrodynamic volume at just over 1.3 ml. This suggests either partial unfolding occurring almost immediately upon addition of urea, or attainment of some equilibrium state of association within the dead time of initiating a gel filtration experiment. Simultaneous addition of both urea and salt similarly caused the protein to elute as a species of hydrodynamic volume larger (≈ 1.51 ml) than that of the dissociated form obtained in 250 mM NaCl (1.6 ml). This suggests either a partial structural destabilization or an association, as in the case of urea alone. That the spectrofluorimetric characteristics did not show any change in 5 M urea, 250 mM NaCl, at this stage, suggested association to be the more likely cause of the increase in volume.

Interestingly, as time passes, there is a very slow inversion of the population to a species eluting with a larger hydrodynamic volume between 1.2 and 1.3 ml. This change was accompanied by the shifting of the fluorescence emission maximum to 352-353 nm; however, whereas the change in fluorescence characteristics was complete by 7 days, the inversion of populations continued to progress beyond this time, and could be seen to be incomplete even after 10 days. Addition of salt to a concentration of 250 mM following 7 days of incubation in 5 M urea (Figure 3.5) elicited nearly the same distribution of the population between states as was observed for 7 days of incubation in the presence of both 5 M urea and 250 mM NaCl. This proves that the effect of urea on the dissociated
Figure 3.4c

Spectrofluorimetric measurements of changes effected in the $\lambda_{\text{max}}$ of the NG-27 xylanase as a function of urea concentration and days of incubation, in the presence of 2 M NaCl. The figure shows that the higher concentration stabilizes the enzyme to such an extent that it cannot be unfolded by urea even after 7 days in the presence of 5-6 M urea.
Fig. 3.5

Abs

with no urea or NaCl

0 hr in 250 mM NaCl

0 hr in 5M urea

0 hr in 5M urea, 250 mM NaCl

1 day

2 days

3 days

7 days

11 days

7 days in 5M urea, 0 hr in 0.25 M NaCl

Elution volume (ml)

Abs$_{280}$
protein is to cause partial unfolding that leads to association to a state similar to that obtained in the absence of salt. Therefore whether salt is added at the beginning of a 7-day incubation with urea, or at the end, makes only as much difference to the apparent kinetics of structural destabilization and association that can be rationalized through the mild hastening of unfolding which appeared to be aided by the presence of NaCl. This is because salt merely effects very rapid dissociation, either of the native form, before urea has effected partial unfolding, or of the unfolded form after urea has effected partial unfolding – on time scales that are very fast. As mentioned previously, both with GdnCl and with the other salts the equilibrium of association-dissociation was found to be achieved within the time taken to mix salt and load a Superdex-200 SMART column. On the other hand, as the fig-2.12 shows, a profound difference could be seen when urea was added at the beginning of a 7-day incubation, rather than at the end of period. This observation clearly established that the unfolding of the dissociated and associated forms of the enzyme are both remarkably slow, with the unfolding of the dissociated form proceeding only somewhat faster. This was also observed to be the case in thermal unfolding experiments.

3.6. KCl and KI enhance enzymatic activity

In further experiment it was found that not only is the enzyme structurally stabilized by multimolar concentrations of KCl, leading to lowering of its susceptibility to thermal unfolding, but also there is an enhancement in the level of enzyme activity at 37 °C. The native-like monomeric form of the xylanase at 2M KCl displayed a 1.4-fold higher activity than the tetrameric form of the xylanase in the absence of salt (Fig.3.6). With potassium iodide (KI), the effect was even more marked, with a nearly 1.9-fold increase in activity accompanying the increase of concentration of the salt to 2 M. Iodide is mildly chaotropic and therefore one would expect it to cause a destabilization of the enzyme’s structure. However, it appeared that the bulk effects exerted by the two halides, KCl and KI are similar in every respect.
Anomalous halophily. The activity of the NG-27 xylanase at 37 °C is enhanced in the presence of salt. Both KCl and KI increase activity progressively with increasing concentration.
Concluding remarks.

The retention of structure under both low-salt and high-salt conditions may be rationalized by the argument that a kinetically-trapped native structure could be responsible for the enzyme’s stability to all sorts of destabilizing conditions, including those which expose it to multi-molar halide solutions. However, the additional stabilization and enhancement of activity by both KCl and KI at such high concentrations can not be so easily rationalized. High concentrations of salt definitely stabilize the monomer.