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A 2D-IR study of heat- and [^{13}C]urea-induced denaturation of sarcoplasmic reticulum Ca^{2+}-ATPase✪

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Two-dimensional infrared correlation spectroscopy (2D-IR) was applied to the study of urea- and heat-induced unfolding denaturation of sarcoplasmic reticulum Ca^{2+}-ATPase (SR ATPase). Urea at 2–3 M causes reversible loss of SR ATPase activity, while higher concentrations induce irreversible denaturation. Heat-induced denaturation is a non-two-state process, with an "intermediate state" (at $t \approx 45^{o}C$) characterized by the presence of protein monomers, instead of the native oligomers. 2D-IR reveals that urea denaturation causes loss of the structural transition to the "intermediate state". Whenever the urea effect can be reversed, the transition to the "intermediate state" is re-established.

Keywords: 2D-IR, heat-induced denaturation, urea-induced denaturation, sarcoplasmic reticulum

The three-dimensional structure of proteins determines their specific actions. This is the basis of the biochemical paradigm of the structure–function relationship. In turn, this paradigm is usually considered to require that the proteins be in their native conformation as a prerequisite for any meaningful biological study. However, this is not necessarily the case, and in fact a deeper understanding of a biological structure is achieved when the transitions between native and non-native forms are studied (Arrondo & Goñi, 1999; Waldner *et al.*, 1999; Glabe, 2004). In the present study, we explore the stability of sarcoplasmic reticulum Ca^{2+}-ATPase using two-dimensional infrared (2D-IR) spectroscopy.

In some simple proteins, denaturing agents, e.g. urea, guanidinium chloride, heat, etc. induce a two-state transition, from native to random conformation (Barone *et al.*, 1997; Mayne & Englander, 2000). However, most proteins, and in particular integral membrane proteins, can be regarded as complex systems containing independent domains that do not necessarily form cooperative structures (Arrondo & Goñi, 1999; Kumar & Yu, 2004). Urea is a chaotropic agent that causes protein unfolding/denaturation by disrupting hydrogen bonds which hold the protein in its unique structure. Urea may also disrupt hydrophobic interactions by promoting the solubility of hydrophobic residues in aqueous solutions (Tanford, 1968; Monera *et al.*, 1994; Soloaga *et al.*, 1998). In turn, heat is probably the most commonly used method to induce protein denaturation. Heat increases the kinetic energy of molecules, thus disrupting hydrogen bonds and hydrophobic interactions (Daniel *et al.*, 1996; Lepock, 2005).

Sarcoplasmic reticulum Ca^{2+}-ATPase (SR ATPase) is an integral membrane protein that pumps calcium out of the cytoplasm during striated muscle relaxation (Sarzala *et al.*, 1982; Martonosi, 1996). This ATPase is part of a family of P-type ion pumps that includes several cation-activated ATPases having in common ten transmembrane helical segments. The structure of SR ATPase has been solved by X-ray crystallography (Martonosi & Pikula, 2003; Toyoshima *et al.*, 2000). The protein consists of a single polypeptide containing, apart from the transmembrane region, a "beak" with an α/β structure, connected to the membrane-spanning domain *via* a mainly α-helical stack.

Infrared (IR) spectroscopy has found extensive use in structure–function studies of membrane

✪Paper dedicated to the memory of Professor Witold Drabikowski and Professor Gabriela Sarzała-Drabikowska.
Abbreviations: IR, infrared spectroscopy; 2D-IR, twodimensional IR spectroscopy; SR, sarcoplasmic reticulum; SR ATPase, SR Ca^{2+}-ATPase.

proteins, among other reasons because the presence of lipids does not interfere with the measurements (for reviews see Arrondo *et al.*, 1994; Arrondo & Goñi, 1999). IR has been used with Ca^{2+}-ATPase to characterize the $E_1 \rightarrow E_2$ conformational transition either by studying the amide I band (Arrondo *et al.*, 1985; 1987; Villalaín *et al.*, 1989) or by using caged compounds (Buchet *et al.*, 1991; Barth *et al.*, 1994). The kinetic mechanism of the enzyme has been described by time-resolved difference spectroscopy using caged ATP (Barth *et al.*, 1996). Thermal denaturation of SR ATPase has been described by Echabe *et al.* (1998) using IR spectroscopy. The process cannot be described as a two-state denaturation, the native oligomers undergoing first a transition to an "intermediate state", probably characterized by the presence of free monomers, that undergo a second transition to unfolded aggregates. The picture that arises from these studies is compatible with early results (Inesi *et al.*, 1973) suggesting that processes governing Ca^{2+} accumulation and ATPase activity are uncoupled at temperatures above 37°C.

More recently, a new approach has been introduced by Noda (for a review see Noda *et al.*, 2000), the so-called generalized 2D-IR correlational spectroscopy. This technique uses correlation analysis of the dynamic fluctuations caused by an external perturbation to enhance spectral resolution without assuming any lineshape models for the bands. This method has been applied to proteins, the perturbation being achieved through changes in temperature, pH, ligand concentration, etc. (Fabian *et al.*, 1999; Contreras *et al.*, 2001; Paquet *et al.*, 2001; Pastrana-Ríos *et al.*, 2002; Turnay *et al.*, 2002; Shanmukh *et al.*, 2002; Torrecillas *et al.*, 2003; Arrondo *et al.*, 2003; Iloro *et al.*, 2004). In the present contribution, 2D-IR is applied to study the effects of heat and urea on the stability of SR ATPase. For this purpose, the amide I band of the IR spectrum, arising mainly from the C=O stretching vibrations of the peptidic bond, was resolved into a number of component bands, each of them corresponding to defined secondary structure elements of the protein. In particular, the changes occurring in the protein structure during the "intermediate state" were characterized. In principle, urea cannot be used in IR studies, because of its strong absorbance in the spectral region corresponding to the protein amide I band. For this reason [^{13}C]urea, whose IR spectrum does not interfere so strongly with the protein amide I band (Reinstädler *et al.*, 1996), was used throughout this study.

MATERIALS AND METHODS

Preparation of sarcoplasmic reticulum vesicles. Sarcoplasmic reticulum vesicles were prepared from rabbit back and leg muscle as described previously (Nakamura *et al.*, 1976; Prado *et al.*, 1983). Sarcoplasmic reticulum Ca^{2+}-ATPase was purified using deoxycholate by method 2 of Meissner *et al.* (1973), yielding closed vesicles essentially free of extrinsic proteins, in which the Ca^{2+}-ATPase accounted for more than 90% of the total protein. Protein concentration was determined by the method of Lowry *et al.* (1951). Ca^{2+}-ATPase activity was assayed as previously described with an ATPase-regenerating system (Prado *et al.*, 1983).

Infrared studies. The protein samples were measured typically at 10 mg/ml in 10 mM Hepes, pH 7.5. The spectra were recorded in a Nicolet Magna II 550 spectrometer equipped with a mercury-cadmium-telluride detector using a demountable liquid cell (Harrick Scientific, Ossining, NY, USA) with calcium fluoride windows, using 6-μm spacers in H_2O medium and 50-μm spacers in D_2O measurements. A tungsten-copper thermocouple was placed directly onto the window and the cell placed into a thermostated cell mount. Typically 1000 scans for each, background and sample, were collected and the spectra obtained with a nominal resolution of 2 cm^{-1}. Data treatment and band decomposition of the original amide I have been described elsewhere (Arrondo *et al.*, 1993; Bañuelos *et al.*, 1995; Arrondo & Goñi, 1999). The mathematical solution to the decomposition may not be unique, but if restrictions are imposed such as the maintenance of the initial band positions in an interval of ≈ 1 cm^{-1}, the preservation of the band width within the expected limits, or the agreement with theoretical boundaries or predictions, the result becomes, in practice, unique.

Urea and thermal treatments. Sarcoplasmic reticulum vesicles at the protein concentration used in the infrared studies were incubated with the desired urea concentrations at room temperature for 5 min. Further incubation did not change the results. After incubation, the vesicles were washed twice by centrifugation at 100000 × *g* for 1 h at 4°C.

Thermal studies were carried out by a continuous heating method. This method uses the Series and Rapid Scan software from OMNIC (Nicolet Corp., Madison, USA). One hundred and sixty-four interferograms/min, using 50 μm pathlength, are collected at 2 cm^{-1} resolution and averaged after each minute. The sample is heated in the interval 20–80°C at 1°C/min. Hence, consecutive spectra correspond roughly to a temperature difference of 1 degree. Temperature is monitored by a probe located at the edge of the window. To obtain the 2D-IR maps, heating was used as the perturbation to induce time-dependent spectral fluctuations and to detect dynamic spectral variations of the secondary structure of SR ATPase. Obtainment of two-dimensional synchronous and asynchronous spectra has been described previously (Turnay *et al.*, 2002).

RESULTS AND DISCUSSION

SR ATPase activity was assessed in SR vesicles in the presence of urea. As seen in Fig. 1, urea at 1 M already causes a dramatic decrease in activity, higher concentrations producing further decreases. When the urea is washed away after treatment, as detailed under Methods, the activity is almost fully recovered for 2 M and 3 M urea. However, at 4 M or 5 M urea appears to cause irreversible damage under our conditions.

The IR spectrum of SR vesicles is shown in Fig. 2A. The amide I band (marked by an asterisk) can be resolved into various component bands (Fig. 2B). Of these, the band centred at about 1658 corresponds to the α-helical components, while the one centred at about 1632 is assigned to β-sheet (Arrondo & Goñi, 1999, and references therein). The change in position of the absorbance maxima of the α and β components during thermal denaturation is shown in Fig. 3. In agreement with the results of Echabe *et al.* (1998), the α-helical component displays a thermal pattern that is close to a typical two-state denaturation, a sigmoid showing a shift in band position in the 48–65ºC range, with a midpoint around 55ºC, very similar to the one obtained for mitochondrial cytochrome *c* oxidase (Arrondo *et al.*, 1994). However, the β-sheet component shows a more complex profile, with a shift in band position from 45ºC to 52ºC, then shifting back between 52ºC and 56ºC. In fact, the α component also shows a very small upshift in the 47–52ºC range. Echabe *et al.* (1998) interpreted these results as suggesting that SR ATPase is an oligomer at room temperature, and, upon temperature increase, it proceeds towards aggregation through a transient monomeric form, the "intermediate state".

Effects of urea, and their reversibility, were observed on the thermal denaturation of SR vesicles (Fig. 4). The thermal profiles of the α-helix and β components in the presence of 3 M urea and after washing it away are shown in Fig. 4. The sample in the presence of urea (empty symbols) lacks the two-state transition (native/denatured), with no signs of an intermediate state either. The α-helix band component appears at a relatively low wavenumber ($\approx$ 1656 cm^{-1}) even at room temperature, and now its position changes with the temperature, moving toward lower values steadily and slowy between about 30ºC and about 60ºC, with no further changes above the latter temperature. It appears as if urea had spread the transition over a wider temperature range, and shifted it to lower temperatures. The thermal profile of the β-sheet component is in itself rather featureless, except that its position at room temperature is at 1635 cm^{-1}, *vs* 1631 cm^{-1} for the native membranes. Heating the 3 M urea-treated samples causes the β-sheet component to shift smoothly to 1637 cm^{-1} at 70ºC (1634 cm^{-1} in native membranes, see Fig. 3). After removing the 3 M urea with two centrifugation steps in urea-free buffer, the complex thermal profile of Fig. 3 is recovered, including the sharp shift of the α-helix at about 55ºC and the intermediate step revealed by the β components (Fig 4A, B, filled symbols). Treatment with 4 M urea also causes virtual disappearance of any sharp transitions

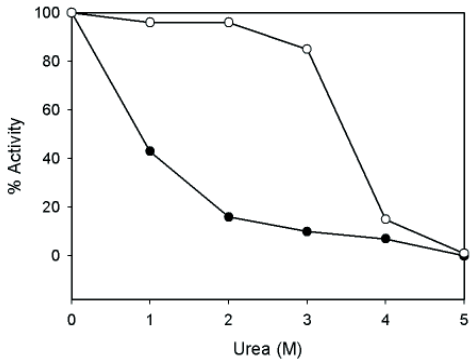

Figure 1. Reversible and irreversible effects of urea on SR ATPase activity.

Enzyme activity was assayed on SR vesicles treated with varying concentrations of urea, before (●) and after (○) washing with urea-free buffer. Average values of two closely similar experiments.

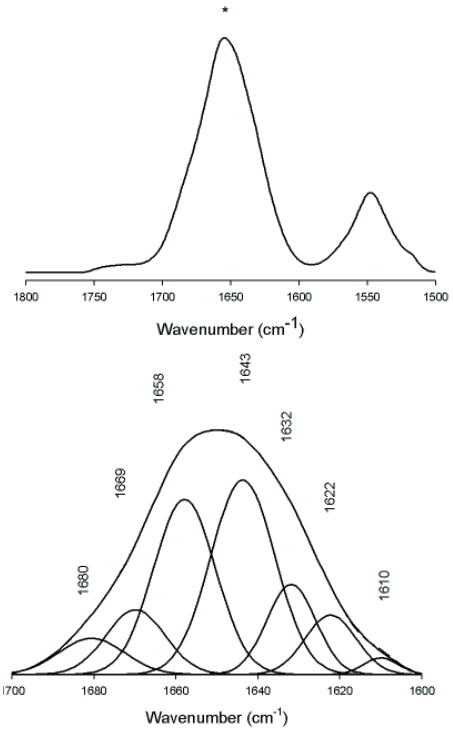

Figure 2. IR spectra of SR.

Top: the 1800–1500 cm^{-1} region of the IR spectrum of purified SR. The band marked with an asterisk (*) is the amide I band, corresponding mainly to peptide carbonyl vibrations. Bottom: the amide I band of SR decomposed into its constituent bands.

that might be associated with thermal denaturation (Fig. 4C, D, empty symbols), but in this case repeated washing does not cause the system to revert to the original situation (Fig. 4C, D, filled symbols). This is in good agreement with the enzyme activity measurements in Fig. 1.

The thermal profiles and curve-fittings shown in Figs. 3 and 4 cannot provide information on the interactions between secondary-structure elements that give rise to the observed changes. Such interactions can, however, be monitored in detail by 2D-IR correlation spectroscopy. In a synchronous 2D map, the peaks located along the diagonal (autopeaks) correspond to changes in intensity induced (in this case) by temperature, and they are always positive. The cross-correlation (non-diagonal) peaks indicate an in-phase relationship between the two bands involved, i.e. document that two vibrations of the protein, characterized by two different wavenumbers (ν_1 and ν_2) are being affected simultaneously (Arrondo *et al.*, 2004). To study the different events taking place during the thermal unfolding of SR ATPase in the presence and absence of urea, correlation maps were obtained, and are shown in Fig. 5.

The 2D-IR study whose results are summarized in Fig. 5 was designed primarily to understand the changes taking place during the "intermediate state" of thermal denaturation, whose onset transition temperature is 45ºC (Fig. 3). For this reason the maps in Fig. 5 were obtained by correlating spectra between 20ºC and 45ºC. The control sample spectral map in the absence of urea is shown in Fig. 5A. Several autopeaks are seen, at 1620, 1632, 1640, 1654, 1664, 1675 and 1682 cm^{-1}, and also a number of cross-peaks, showing an important redistribution of the secondary structures. The fact that 4 M and higher concentrations of urea lead to irreversible structural and functional changes in SR ATPase indicates a profound modification of protein conformation, including likely the transmembrane domain. However, in the presence of urea no differences are seen in this temperature region, indicating that the intermediate state (characterized by band changes at about 1640 cm^{-1}) is lost under these conditions, in agreement with the thermal profiles.

Figure 5B was obtained for a sample in 3 M urea. There is only background noise, and the

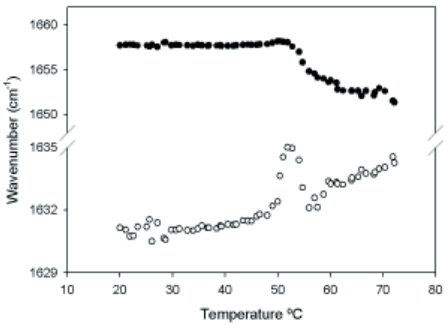

Figure 3. Thermal unfolding of SR.

Temperature-dependent shifts of band components corresponding to α-helix (●) and β-sheet (○).

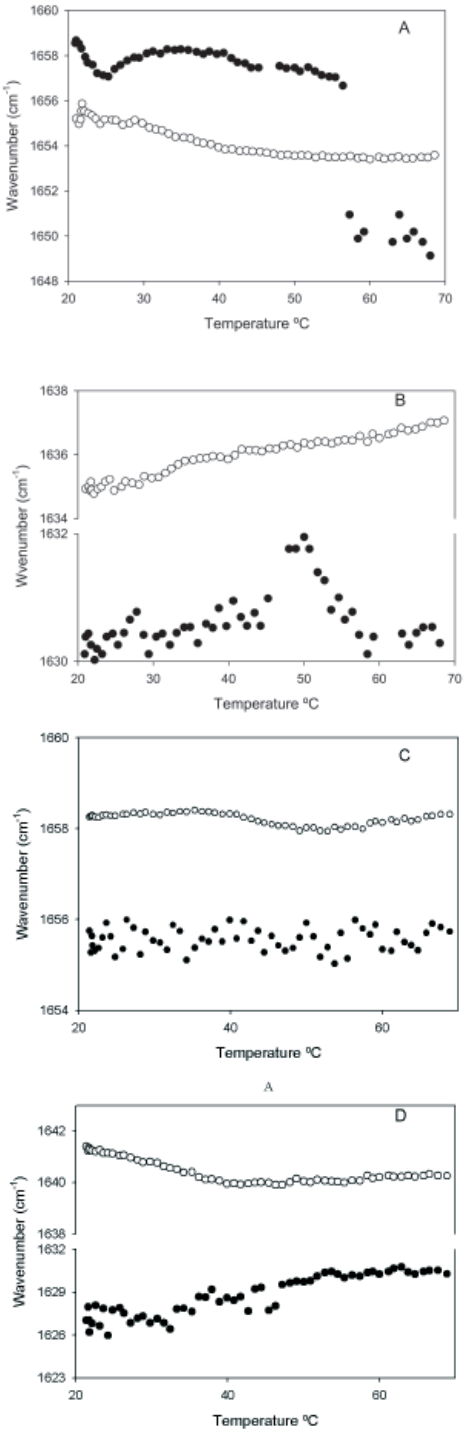

Figure 4. Reversibility of urea effects on the thermal unfolding of SR.

Temperature-dependent shift of band components corresponding to α-helix (A, C) and β-sheet (B, D). Urea concentration was 3 M (A, B) and 4 M (C, D). Spectra recorded after urea treatment, before (○) and after (●) washing with urea-free buffer.

characteristic shift of the urea band "tail". So 2D-IR confirms the lack of an intermediate transition during SR ATPase thermal denaturation in the presence of 3 M urea. The reversibility of the 3 M urea effect is apparent in Fig. 5C, obtained for SR vesicles treated with 3 M urea, then washed. The original pattern (Fig. 5A) is almost quantitatively recovered. Also in agreement with our previous observations, treatment with 4 M urea prevents the oligomer–monomer transition of the enzyme, so that the corresponding spectral map is featureless (Fig. 5D), and this effect cannot be reverted by urea removal (Fig. 5E). The recovery of the correlational maps in 3 M urea, but not in 4 M urea points again to a perturbation of the intramembrane domain of the ATPase at the higher urea concentrations.

The results presented include a combination of the classical infrared approach, using band decomposition and thermal profiles, with correlational 2D-IR spectroscopy. Temperature is the perturbing agent in this study, but other parameters can be used in the same way, e.g. the presence of lipids (Shanmukh *et al.*, 2003, Torrecillas *et al.*, 2003) or other external ligands (Pastrana-Rios *et al.*, 2001). As a result, 2D-IR provides more detailed and informative spectra than the conventional infrared approach. In the work presented, 2D-IR spectroscopy allowed

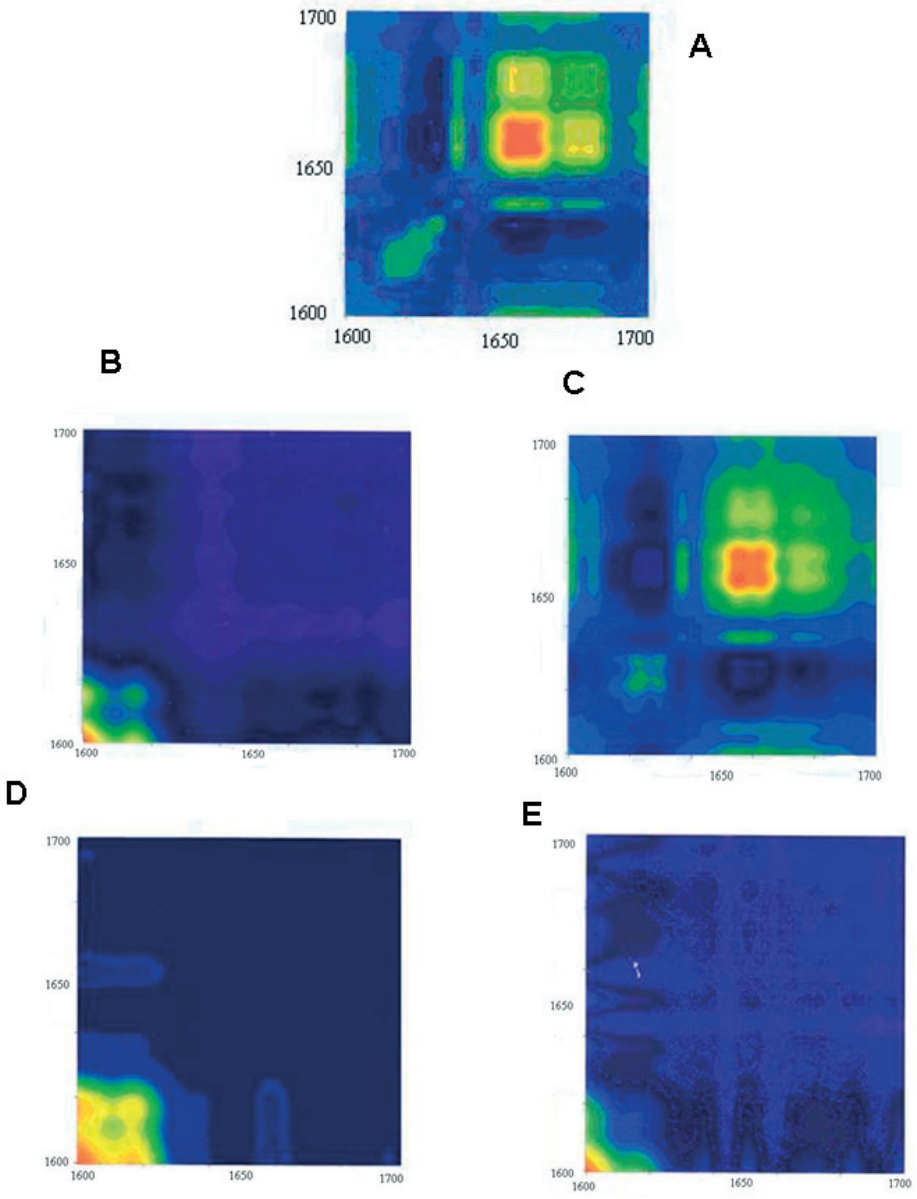

Figure 5. 2D-IR synchronous correlation spectra of IR.

Spectra were obtained by correlating data in the interval 20–45°C (t_m of the intermediate state). (A) Control, non-urea treated. (B) 3 M urea, (C) 3 M urea, then washing twice in urea-free buffer. (D) 4 M urea, (E) 4 M urea, then washing twice in urea-free buffer.

us to easily discern the changes produced in the sarcoplasmic reticulum by the presence of urea and by its eventual removal.

CONCLUSIONS

1. The chaotropic agent urea at 2–3 M concentrations causes a decrease of the SR ATPase activity to about 20% of the original value. After urea removal by two steps of washing and centrifugation the activity recovers up to 85–90% of its native value.

2. Conventional and 2D-IR spectroscopy reveal that urea at 2–3 M perturbs α and β structures in the protein, which not only causes the loss of enzyme activity, but also prevents the thermal transition from the native to the "intermediate structural state". These structural effects are reverted after urea removal.

3. Urea concentrations above 3 M perturb the protein structure in a non-reversible way. This is accompanied by an equally irreversible loss of enzyme activity.

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