Perchlorate-Induced Denaturation of Ribonuclease A: Investigation of Possible Folding Intermediates†

J. Martin Scholtz and Robert L. Baldwin*

Department of Biochemistry, Stanford University School of Medicine, Stanford, California 94305-5307

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ABSTRACT: Perchlorate-denatured ribonuclease A (PDR) is known to show a circular dichroism (CD) spectrum suggestive of substantial secondary structure. Thus, PDR may be a molten globule form of ribonuclease A. We find that any secondary structure present in PDR does not provide measurable protection against amide proton exchange, and PDR does not belong to the class of structured molten globules. CD spectra of short peptides show that the perchlorate anion affects these spectra in a way that could be mistaken for secondary structure formation; thus, caution must be used in interpreting CD spectra of peptides and proteins taken in perchlorate solutions.

The identification and characterization of protein folding intermediates may help shed light on one of the fundamental problems remaining in biochemistry: the protein folding process (Kim & Baldwin, 1990). In recent years, there have been two major approaches used to study folding intermediates. One is to look at kinetic intermediates on the refolding pathway for a number of proteins using amide proton exchange and rapid-mixing techniques [for reviews, see Baldwin and Roder (1991) and Baldwin (1993)]. The other approach has been to study equilibrium intermediates, termed compact intermediates or molten globules, which are populated under conditions extreme in pH and/or ionic strength [for review, see Kuwajima (1989)]. There is growing evidence that these equilibrium, molten globule intermediates may be on the kinetic pathway for protein refolding (Kuwajima, 1989; Ptitsyn et al., 1990; Baldwin, 1993, and references therein), and thus a detailed characterization of the structures of molten globule intermediates is critical for our understanding of protein folding.

Ribonuclease A (RNase A)[1] from bovine pancreas has been the subject of many protein folding and stability studies over the years. The refolding has been studied by classical spectroscopic techniques (Hagerman & Baldwin, 1976) as well as by amide proton exchange using rapid-mix and quench techniques (Udgaonkar & Baldwin, 1988, 1990). Our interest in identifying potential equilibrium folding intermediates for RNase A prompted us to investigate the effects of perchlorate salts on the structure of RNase A at low pH. It was shown by Ahmad and Bigelow (1979) that the addition of $LiClO_4$ to RNase A at low pH resulted in a cooperative transition to a form of RNase A that retains approximately 90% of the secondary structure of native RNase A as measured by CD. Furthermore, Ahmad and Bigelow (1979) were able to demonstrate that the perchlorate form of RNase A could be cooperatively denatured with the addition of a second denaturant, in this case urea. In related studies, Denton et al. (1982) and Lynn et al. (1984) showed that, although the perchlorate-denatured form of RNase A retains significant structure, the refolding kinetics of the perchlorate-denatured form and the urea-denatured form of RNase A are very similar, suggesting that the perchlorate-induced intermediate either is not on the kinetic pathway for folding or else is formed in the burst phase of stopped-flow refolding. The equilibrium molten globule intermediate, or a very similar species, is formed in the burst phase of stopped-flow refolding of α-lactalbumin (Ikeguchi et al., 1986), and the same is true of apomyoglobin (Jennings & Wright, 1992).

The main focus of this report is to characterize further the structure of the perchlorate-denatured form of RNase A and to determine if it is indeed a molten globule. We find that, although RNase A retains a large CD signal in the presence of perchlorate salts, there is not significant protection from amide proton exchange, indicating that any hydrogen-bonded secondary structure is not stable. We also wish to report the effects of perchlorate salts on the CD characteristics of several small peptides in hopes of explaining the CD spectral characteristics of RNase A in perchlorate solutions.

MATERIALS AND METHODS

Materials. Bovine pancreatic RNase A (type XIIA, Sigma) was further purified by ion-exchange chromatography as described previously (Garel, 1976). RNase A concentration was determined using the molar extinction coefficient 9800 M^{-1} cm^{-1} at 278 nm (Sela & Anfinsen, 1957). Reagent grade buffers and salts were obtained from J. T. Baker, Inc. D_2O and DCl were from Cambridge Isotope Laboratories.

All peptides were synthesized using solid-phase peptide synthesis procedures and were purified using HPLC as described previously (Scholtz et al., 1991). The N-terminus and the C-terminus were blocked with acetyl and carboxamide, respectively. The purified peptides were homogeneous as determined by HPLC and gave the expected molecular weight of the parent ion as determined by FAB mass spectroscopy.

Circular Dichroism Measurements. CD spectra for RNase A were taken at 25 °C in 50 mM glycine with 100 mM LiCl, pH 3.0, and the indicated amount of $LiClO_4$. CD spectra for the peptides were obtained at pH 3.0 in the same buffer as that for RNase A or at pH 7.5 in 10 mM sodium phosphate buffer with 100 mM NaCl or LiCl and the indicated amount of sodium or lithium perchlorate. Peptide concentration was

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* To whom correspondence should be addressed.

[1] Abbreviations: NMR, nuclear magnetic resonance; HPLC, high-performance liquid chromatography; FAB, fast atom bombardment; CD, circular dichroism; FID, free induction decay; RNase A, bovine pancreatic ribonuclease A. The one-letter codes for amino acids and the abbreviations for the terminal blocking groups are as follows: A; alanine; D, aspartic acid; H, histidine; K, lysine; Y, tyrosine; Ac, acetyl; NH_2, carboxamide.

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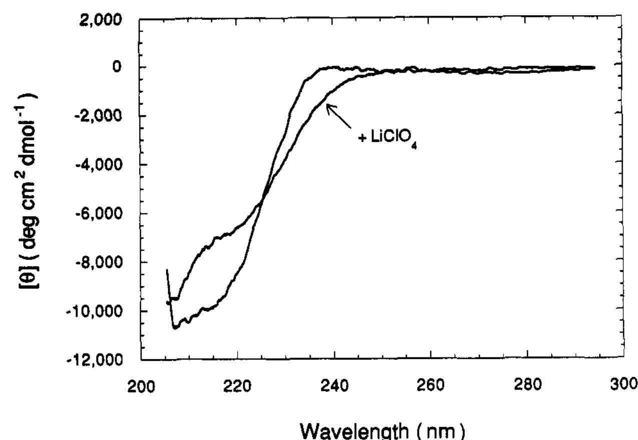

FIGURE 1: CD spectra of RNase A in the presence and absence of 3.0 M $LiClO_4$ at 25°C. Each buffer contains 50 mM glycine, 100 mM LiCl, pH 3.0, and 6 μM RNase A.

determined by measuring tyrosine absorbance at 275 nm in 6 M guanidine hydrochloride and 20 mM potassium phosphate, pH 6.5 (Brandts & Kaplan, 1973), for the tyrosine-containing peptides, or by quantitative amino acid analysis using ninhydrin (Rosen, 1957). An Aviv 60DS spectropolarimeter with a Hewlett-Packard 89100A temperature control unit and cuvettes with either 10- or 1-mm path lengths were used for all measurements. Ellipticity was calibrated with (+)-10-camphorsulfonic acid and is reported as mean molar residue ellipticity, [θ] (deg cm^2 dmol^{-1}).

NMR Spectroscopy. Samples were prepared by dissolving RNase A in water to a concentration of 1–3 mM and adjusting the pH to 3.0 with HCl. The samples were then lyophilized to dryness. To initiate the H–D exchange reaction, the protein was dissolved at 0.5–3 mM in D_2O at 25 °C containing 25 mM glycine plus either 100 mM NaCl or 100 mM LiCl and the indicated amount of the perchlorate salt, pH* 3.0. pH* represents the glass electrode reading at room temperature, uncorrected for isotope effects. All reported pH* measurements were made after the acquisition of the NMR spectra. After the protein was dissolved in buffer at $t = 0$ min, multiple one-dimensional proton spectra were acquired during the exchange on a General Electric GN-Omega spectrometer at a proton frequency of 500.13 MHz. Data were collected using a 6000-Hz spectral width and a 60° pulse at 25 °C. The FID was the sum of 32 scans collected in 4096 complex points with a 5-ms recycle delay. The acquisition of each spectrum required approximately 2 min. Spectra were processed using FELIX (Hare Research, Inc.) on a Silicon Graphics Personal Iris computer. FIDs were multiplied by a exponential decay function with line broadening of 2 Hz before Fourier transformation. Proton occupancy was determined by measuring the integral of all peaks in the amide region of each spectrum and normalizing to the area of nonexchanging aromatic tyrosine resonances to correct for differences in spectrum plotting.

RESULTS

Perchlorate Effects on the Structure of RNase A. Figure 1 shows the effect of $LiClO_4$ on the CD spectrum of RNase A at pH 3.0. The addition of 3 M $LiClO_4$ does not produce a spectrum characteristic of an unfolded, random-coil polypeptide (Woody, 1985); rather a substantial CD signal at wavelengths above 225 nm persists. The increased ellipticity is not a function of the buffer itself, as blank solutions (without protein) give identical CD signals with and without perchlorate.

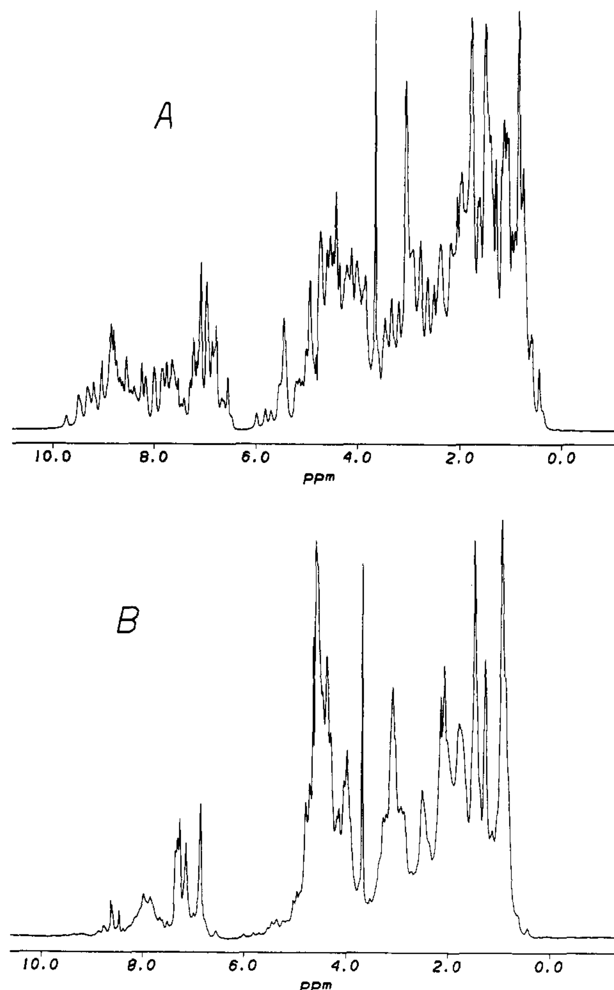

FIGURE 2: (A) 1-D NMR spectrum of RNase A in 25 mM glycine and 100 mM LiCl, 25°C, pH* 3.06, immediately after the protein was dissolved in D_2O. (B) 1-D NMR spectrum of RNase A in 25 mM glycine, 100 mM LiCl, and 3.0 M $LiClO_4$, 25 °C, pH* 3.04, immediately after the protein was dissolved in D_2O. The signal at $\delta = 3.27$ ppm results from the α-protons of the glycine buffer.

The effect is also not limited to $LiClO_4$, as $NaClO_4$ produces a similar effect, although a higher concentration of $NaClO_4$ is required (data not shown). The persistence of ≈90% of the CD signal at 225 nm in the presence of $LiClO_4$ has been demonstrated in an earlier report by Ahmad and Bigelow (1979) and by Denton et al. (1982). Ahmad and Bigelow also observed a cooperative transition with the addition of $LiClO_4$ [see Figure 1 of Ahmad and Bigelow (1979)]. We observe similar cooperative denaturation transitions of RNase A at pH 3.0 with both $LiClO_4$ and $NaClO_4$ (data not shown). We elected to use 3.0 M $LiClO_4$ at pH 3 to induce the perchlorate-denatured form of RNase A in order to compare our results with those of Ahmad and Bigelow (1979).

Furthermore, Ahmad and Bigelow (1979), as well as Denton et al. (1982), were able to show that the addition of urea to the perchlorate form of RNase A produced a second cooperative denaturation transition which results in a CD spectrum which resembles that expected for a random-coil polypeptide. From their data, they are able to produce phase diagrams which describe the stability of RNase A to denaturation by urea and lithium perchlorate. We are able to reproduce these results using CD as a means to monitor the amount of secondary structure present in RNase A.

The structure of RNase A in solutions with a high perchlorate concentration was also investigated by NMR.

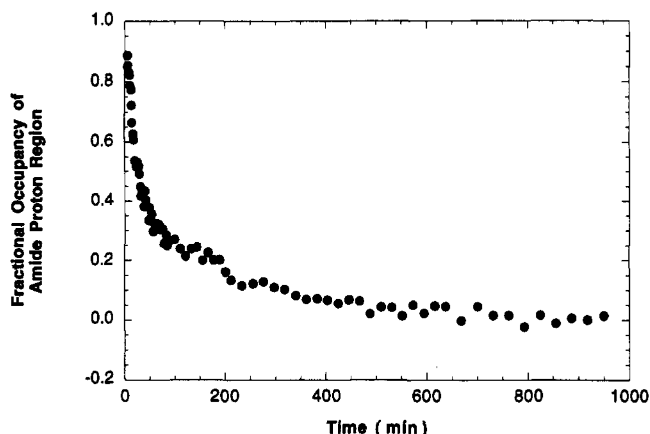

FIGURE 3: The amide proton H–D exchange curve for the perchlorate-denatured form of RNase A as measured by NMR. The protein was dissolved in D_2O containing 25 mM glycine, 100 mM NaCl, and 4.0 M $NaClO_4$, pH* 3.02 at 25 °C. After various exchange-out times, a one-dimensional NMR spectrum was recorded and the occupancy of the amide proton region was determined as described in Materials and Methods.

Figure 2 shows one-dimensional NMR spectra of RNase A, freshly dissolved in D_2O buffer, in the absence (Figure 2A) and presence (Figure 2B) of $LiClO_4$ at low pH. The addition of perchlorate reduces the chemical shift dispersion found in fully folded RNase A and gives rise to broader lines in the NMR spectrum. The general appearances of the spectra are independent of protein concentration between 0.5 and 3.0 mM, suggesting that concentration-dependent aggregation is not occurring. The NMR spectrum of the perchlorate form of RNase A resembles that found for random-coil polypeptides, although many molten globule forms of other proteins have NMR spectra that appear to be similar to unfolded proteins (Dolgikh et al., 1985; Griko et al., 1988; Baum et al., 1989).

In order to determine if the perchlorate-denatured form of RNase A contains hydrogen-bonded secondary structure, as suggested by the large residual CD signal, the bulk amide proton exchange behavior was measured under the same conditions as those used in the CD characterization. The results, which are shown in Figure 3, suggest that there is no significant reduction in the bulk H–D exchange rate. The half-time for complete exchange of the amide protons in the perchlorate-denatured form of RNase A is approximately 35 min. This half-life for H–D exchange is about 4 times that predicted for poly-(D,L-alanine) (Molday et al., 1972; Englander & Poulsen, 1969). It is known that the addition of salt can affect the intrinsic exchange rates of unstructured polypeptides (Kim & Baldwin, 1982); however, the correction for the effect of nonspecific salt would be small for the conditions of our experiments and would make our exchange rates faster still. Furthermore, the observed exchange rate for perchlorate-denatured RNase A is nearly identical to that observed for oxidized RNase A (Englander, 1963; Molday et al., 1972) when normalized to the same conditions. The oxidized derivative of RNase A is thought to be completely devoid of any structure. The agreement between the H–D exchange curves for these two forms of RNase A suggests that there is no significant hydrogen-bonded structure in the perchlorate-denatured form of RNase A in spite of the large signal in the far-UV CD spectrum.

Perchlorate Effects on Short Peptides. Since we have found that perchlorate salts appear to produce a large CD signal in denatured RNase A, we decided to investigate the effects of perchlorate salts on short peptides that cannot adopt organized structures. We chose three different peptides containing primarily alanine with a single lysine, histidine, or aspartic acid residue in order to determine the importance of the charge on the peptide. The single tyrosine in two of the peptides facilitates peptide concentration determination. Figure 4 shows the effect of perchlorate (Li^+ or Na^+ as the cation) on the CD spectrum of Ac-AAKAAY(NH_2), the peptide with a single lysine residue. The addition of perchlorate produces an increase in the negative ellipticity at wavelengths above 210 nm. There does appear to be a difference in the magnitude of the CD signal below 200 nm for the two different cations. This may be due to a specific Li^+ effect on the peptide amide chromophore (J. A. Schellman, personal communication). Similar results were found for the effect of perchlorate salts on two peptides with different charged side chains: Ac-AHAY(NH_2) at pH 7.5 and 3.0 and Ac-AADAA(NH_2) at pH 3.0 and pH 7.5. Figure 5 shows the effect of $NaClO_4$ on the peptide containing aspartic acid at low pH where the peptide contains no charged side chains. The inset shows the change in ellipticity at 222 nm with increasing $NaClO_4$ concentration. In all of these cases, a substantial CD signal above 210 nm is induced in the presence of perchlorate salts. Although the magnitude of the increased CD signal is not identical for all three peptides, the signal does not depend on

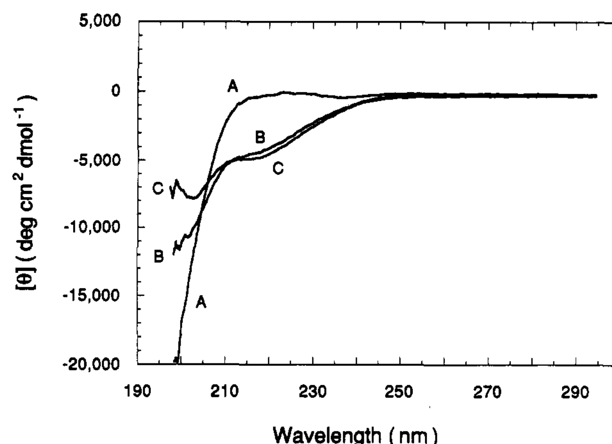

FIGURE 4: Perchlorate effects on the CD spectrum of a six-residue, random-coil peptide, Ac-AAKAAY(NH_2), at 25 °C. Each sample is buffered at pH 3.0 with 25 mM glycine and 100 mM LiCl or NaCl. Spectrum A is in the absence of perchlorate; spectrum B is in the presence of 3.0 M $LiClO_4$; spectrum C is in the presence of 4.0 M $NaClO_4$.

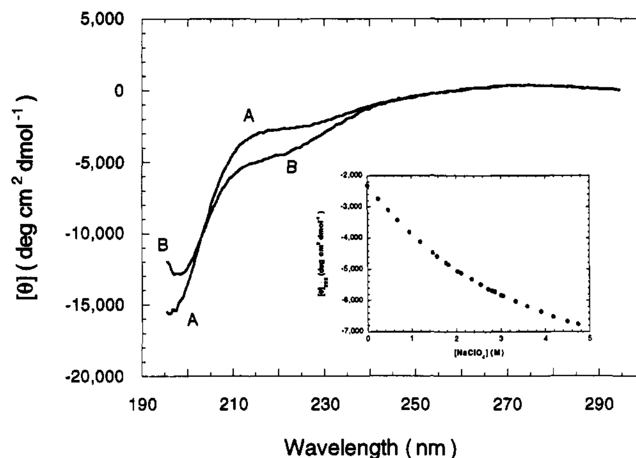

FIGURE 5: Perchlorate effects on the CD spectrum of a five-residue, random-coil peptide, Ac-AADAA(NH_2), at 25 °C and pH 2.5 (100 mM NaCl and 10 mM sodium phosphate buffer) in the absence of perchlorate (A) and in the presence of 2.0 M $NaClO_4$ (B). The inset shows the effect of $NaClO_4$ on the mean residue ellipticity at 222 nm under the same conditions.

the pH of the solution, the nature of the peptide, or the cation (Li^+ or Na^+) of the perchlorate.

DISCUSSION

The Perchlorate-Denatured Form of RNase A Is Not a Structured Molten Globule. In spite of the large residual CD spectrum and the fact that perchlorate salts cooperatively denature RNase A, the remaining structure does not appear to meet the criteria for a structured molten globule intermediate, which should have a substantial amount of stable hydrogen-bonded secondary structure without any measurable tertiary structure. The NMR spectrum of the perchlorate-denatured form of RNase A is consistent with the lack of defined tertiary structure and appears to be that expected for a random-coil polypeptide, although many molten globule intermediates appear to have NMR spectra that are similar to denatured proteins (Dolgikh et al., 1985; Griko et al., 1988; Baum et al., 1989). Our main evidence against a molten globule form for RNase A comes from the lack of observable protection from amide proton exchange. It has been shown (Rohl et al., 1992) that substantial protection from H–D exchange can be provided by helical structure even in an isolated α-helix. We are unable to observe any hydrogen-bonded secondary structure using amide proton exchange and NMR under the same conditions in which a large CD signal is observed. Therefore, we must conclude that the perchlorate-denatured form of RNase A is not a molten globule, but rather a structureless denatured protein.

The similarities between these results and those obtained for thermally unfolded RNase A (Robertson & Baldwin, 1991) are striking. Both the perchlorate-denatured and thermally denatured forms of RNase A have large, residual CD signals that are different from those found for the urea- or guanidinium chloride-denatured forms of RNase A. In both cases, however, no substantial protection from exchange of the amide protons with solvent was observed. It appears that in the case of both thermally denatured and perchlorate-denatured RNase A the CD signal in the far-UV is not reporting on any stable, hydrogen-bonded secondary structure. Thermally denatured RNase A is, however, more compact than the random-coil form, as measured by dynamic light scattering (Nicoli & Benedek, 1976), and Fourier transform infrared data (Seshadri et al., 1993) support the suggestion from the CD spectrum that considerable hydrogen bonding of the peptide NH groups is present in thermally denatured RNase A.

Perchlorate Salts Affect the CD Spectra of Peptides. In order to understand the far-UV CD spectrum of denatured RNase A in the presence of perchlorate salts, we chose to look at the effects of perchlorates on small, unstructured peptides. We selected small peptides (4–6 residues) to minimize the possibility that the peptides might adopt any specific structure, in order to investigate the effects of perchlorate salts on the CD spectra of random-coil peptides. The peptides contain various charged side chains ranging from lysine and histidine to aspartic acid. The perchlorate effect on the CD signal does not depend on the nature of the charged side chain or the pH of the solution.

Perchlorate salts are known to affect the CD behavior and structure of polymeric peptides containing cationic side chains. Bello (1988, 1992) has investigated the effects of perchlorate salts on poly(lysine) and poly(N^ϵ,N^ϵ,N^ϵ-trimethyl)lysine derivatives. He finds that perchlorates are able to convert the random-coil form of the polymer to an α-helical form at neutral pH. The same behavior has been demonstrated for other polymers of cationic residues, namely, the polymers of arginine (Rifkind, 1969) and ornithine (Corio et al., 1974; Saito et al., 1978; Bello et al., 1985). The results presented by Bello (1988, 1992) for poly(lysine) and the lysine derivative are interpreted with a simple model for binding of the perchlorate anion to the cationic side chains and not with an interaction between the perchlorate and the peptide backbone. Our results do not support this simple binding model for our peptides, as cationic, anionic, and neutral peptides exhibit very similar CD behavior with increasing perchlorate concentration.

One of the observations made by Bello (1988) for both poly(lysine) and the trimethylated derivative in 5.4 M $NaClO_4$ is that a substantial amount of CD signal remains at high temperatures. He suggests two roles for $NaClO_4$: at moderate concentrations, it acts as a denaturant and disrupts the α-helical structure in the polypeptides, while at higher concentrations it exhibits a general salt effect which promotes hydrophobic interactions. Our results are consistent with this dual role for perchlorate salts. The hydrophobic effect caused by high concentrations of perchlorate will produce a more compact structure in RNase A and, perhaps, lead to an increase in the far-UV CD signal without the formation of hydrogen-bonded secondary structure.

A General Caution about Perchlorate Salts and CD. Our results indicate that peptides or proteins in high concentrations of perchlorate salts give far-UV CD spectra suggestive of secondary structure formation, while a second probe for structure formation is unable to detect any stable structure. The exact nature of the interaction of perchlorate salts with polypeptides cannot be determined from our results; it does seem clear, however, that using CD alone as a probe of secondary structure may not give a measure of the amount of structure present.

One of the ways in which a molten globule form has been induced in many proteins is by using perchlorate salts at low pH. Goto et al. (1990) have investigated the effects of many different anions on the acid-induced unfolding of apomyoglobin and cytochrome *c*. For these proteins, the unfolding transitions were monitored by CD. It does not appear that perchlorate is behaving abnormally with respect to its position in the Hofmeister series (Collins & Washabaugh, 1985) or in the electroselectivity series of anions found by anion-exchange resins (Gregor et al., 1955; Gjerde et al., 1980). In a related study, Goto and Aimoto (1991) have looked at the effects of perchlorate salts on the conformation of a model polypeptide which forms a helical structure in the presence of different salts as monitored by far-UV CD. Again, perchlorate salts appear to behave in the manner expected, based on their solution properties. In both studies, the concentrations of perchlorate salts were well below that used in this report; this may explain the apparent contradiction.

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