been shown to disrupt the binding and functional coupling of F_1 to F_0 (Mosher et al., 1985; Miller et al., 1989), and the simplest explanation is to place these residues at the F_0–F_1 interface. This interpretation is supported by the experiments presented here, where an epitope in the $Lys_{34} \rightarrow Ile_{46}$ segment of the protein was placed at the F_1-binding surface of the membrane.

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SUPPLEMENTARY MATERIAL AVAILABLE

Details of the $Lys_{34} \rightarrow Ile_{46}$ peptide synthesis and purification (4 pages). Ordering information is given on any current masthead page.

REFERENCES

Barany, G., & Merrifield, R. B. (1980) in *The Peptides: Analysis, Synthesis, Biology* (Gross, E., & Meienhofer, I., Eds.) Vol. 2, pp 1–284, Academic Press, New York.

Beechey, R. B., Linnett, P. E., & Fillingame, R. H. (1979) *Methods Enzymol. 55*, 426–434.

Briand, J. P., Muller, S., & Van Regenmortel, M. H. V. (1985) *J. Immunol. Methods 78*, 59–69.

Deckers-Hebestreit, G., & Altendorf, K. (1986) *Eur. J. Biochem. 161*, 225–231.

Deckers-Hebestreit, G., Schmid, R., Kiltz, H.-H. & Altendorf, K. (1987) *Biochemistry 26*, 5486–5492.

Fillingame, R. H. (1976). *J. Biol. Chem. 251*, 6630–6637.

Fillingame, R. H., Mosher, M. E., Negrin, R. S., & Peters, L. K. (1983) *J. Biol. Chem. 258*, 604–609.

Fillingame, R. H., Porter, B. P., Hermolin, J., & White, L. K. (1986) *J. Bacteriol. 165*, 244–251.

Foster, D. L., & Fillingame, R. H. (1982) *J. Biol. Chem. 257*, 2009–2015.

Goodfriend, T. L., Levine, L., & Fasman, G. D. (1964) *Science 144*, 1344–1346.

Graf, T., & Sebald, W. (1978) *FEBS Lett. 94*, 218–222.

Hermolin, J., & Fillingame, R. H. (1989) *J. Biol. Chem. 264*, 3896–3903.

Hermolin, J., Gallant, J., & Fillingame, R. H. (1983) *J. Biol. Chem. 258*, 14550–14555.

Hopp, T. P., & Woods, K. R. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 3824–3828.

Hoppe, J., & Sebald, W. (1984) *Biochim. Biophys. Acta 768*, 1–27.

Hoppe, J., Brunner, J., & Jorgensen, B. B. (1984) *Biochemistry 23*, 5610–5616.

Loo, T. W., & Bragg, P. D. (1982) *Biochem. Biophys. Res. Commun. 106*, 400–406.

Miller, M. J., Fraga, D., Paule, C. R., & Fillingame, R. H. (1989) *J. Biol. Chem. 264*, 305–311.

Mosher, M. E., White, L. K., Hermolin, J., & Fillingame, R. H. (1985) *J. Biol. Chem. 260*, 4807–4814.

Senior, A. E. (1988) *Physiol. Rev. 68*, 177–231.

Folding of a Peptide Corresponding to the α-Helix in Bovine Pancreatic Trypsin Inhibitor†

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ABSTRACT: A short peptide corresponding to the α-helical region of BPTI shows partial folding in aqueous solution (pH 7) as judged by circular dichroism (CD). Folding is temperature and denaturant sensitive, and the peptide is monomeric. The difference CD spectrum, obtained from spectra at two temperatures, indicates that the peptide folds as an α-helix. Difference CD spectroscopy provides a sensitive assay for helix formation in peptides exhibiting small amounts of structure. Helix stability in this peptide shows a marked pH dependence which is consistent with stabilizing charged side-chain interactions with the helix dipole and/or salt bridge formation.

Since proteins in general contain sufficient information in their amino acid sequence to enable them to fold spontaneously, it is interesting to ask how much of a protein is required for structure to occur. In other words, can part of a protein (a peptide) exhibit autonomous folding into native-like structure? There are now a few examples of aqueous, monomeric "autonomous folding units" (AFUs). C-Peptide, corresponding to an α-helical region in ribonuclease A, shows ~30% α-helical structure at 0 °C in aqueous solution (Brown & Klee, 1971; Bierzynski et al., 1982; Shoemaker et al., 1987). A nine-residue peptide with a native sequence adopts a reverse turn structure in aqueous solution (Dyson et al., 1985), and a peptide corresponding to another native helical sequence exists as a collection of transient α-helical turns in aqueous solution (Dyson et al., 1988). Short peptides designed de novo with several $i, i + 4$ type salt bridges have as much as ~80% helix content at 0 °C (Marqusee & Baldwin, 1987).

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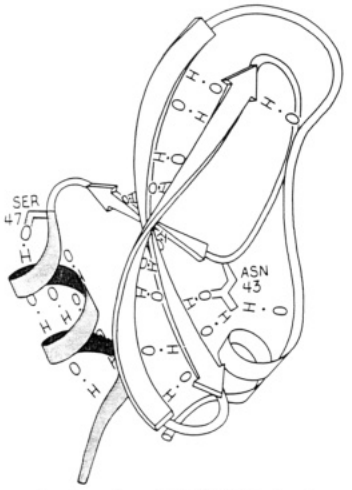

FIGURE 1: Schematic drawing of BPTI (Richardson, 1985) showing the α-helical region corresponding to the peptide Pα5.

The existence of AFUs is implied by several models describing protein folding and structure. The framework model postulates that small isolated units of secondary structure form early in the folding process and then coalesce to form stabilizing tertiary interactions [see review by Kim and Baldwin (1982)]. The molten globule state observed for some proteins (under conditions of low pH or moderate concentrations of denaturant) is believed to arise from intact secondary structure in the absence of fixed tertiary structure (Ptitsyn, 1987). The theory of exon shuffling (Gilbert, 1978, 1985) proposes that new proteins can be made by recombining exons and suggests that such exons code for AFUs.

The results presented here provide another example of an AFU: the peptide Pα5 (corresponding to the α-helical region of BPTI) is shown to have partial helical structure at low temperature in aqueous solution. Pα5 corresponds to residues 47–58 of BPTI and is shown as the shaded region of BPTI in Figure 1. The two cysteine residues (Cys-51 and Cys-55) in this region of native BPTI have been replaced with Ala, and the N-terminus is acetylated. The sequence of Pα5 (using BPTI numbering) is

AcSer-Ala-Glu-Asp-Ala-Met-Arg-Thr-Ala-Gly-Gly-Ala
47 48 49 50 51 52 53 54 55 56 57 58

A second peptide, Pα7, which is identical in sequence but contains a free N-terminus, shows less helix content than Pα5 [cf. Shoemaker et al. (1987)].

Materials and Methods

The peptides were synthesized using solid-phase *t*-Boc methods [see review by Kent (1988)] on an Applied Biosystems Model 430A peptide synthesizer and were cleaved from the resin using TFMSA (Yajima & Fujii, 1983; Tam et al., 1986). The peptides were desalted on a Sephadex G-10 column in 5% acetic acid and purified by reversed-phase HPLC on a Vydac C18 semipreparative column using a linear water/acetonitrile gradient containing 0.1% TFA. Confirmation of the peptide identity was obtained at the MIT mass spectrometry facility, which gave the expected molecular ions of 1178 for Pα5 and 1137 for Pα7.

The peptide concentration of stock solutions was determined after alkaline hydrolysis by the ninhydrin method, using leucine as a standard (Rosen, 1957), and the estimated error was $\pm 12\%$ (2σ). Acid instead of alkaline hydrolysis gave a similar concentration for Pα5. CD samples were prepared by diluting these stock solutions into a 0.1 M NaCl/1 mM Na_2HPO_4 buffer of the indicated guanidine hydrochloride (Gdn-HCl) concentration. In all CD experiments (except for the concentration dependence studies), the concentration of Pα5 was 0.12 mM, and that of Pα7 was 0.10 mM. The pH of the solutions was adjusted at room temperature using HCl and NaOH. For the pH dependence of the CD signal experiment, the buffer used was 0.1 M NaCl, 10 mM Na_2HPO_4, and 10 mM NaOAc. The pH was measured at room temperature before and after the CD experiment, and the change in pH was <0.1.

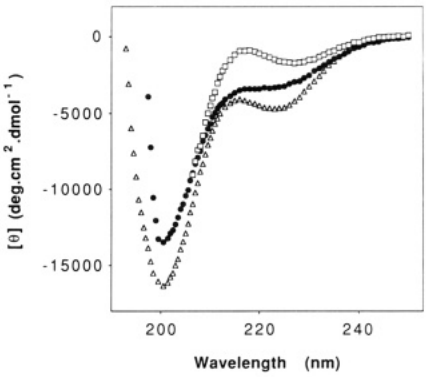

FIGURE 2: CD spectra of Pα5 in 0.1 M NaCl/1 mM Na_2HPO_4, pH 7, at 0 °C, 0 M Gdn-HCl (△); 60 °C, 0 M Gdn-HCl (●); 0 °C, 2 M Gdn-HCl (□).

CD experiments were performed on an AVIV Model 60DS CD spectrometer using a 1-mm path-length cell for spectra and a 10-mm cell for other experiments. The temperature was controlled by a water bath for the 1-mm cell and by a HP Model 89100A Peltier temperature-control unit for the 10-mm cell. All samples were degassed prior to use.

Gel filtration results were kindly provided by Dr. T. G. Oas (Oas & Kim, 1988). A Sephadex G-25 column (1.6 × 40 cm) in 0.2 M Na_2SO_4/10 mM Na_2HPO_4 at pH 6 and at 4 °C was used. The use of this buffer instead of that used for the CD experiments did not alter the CD characteristics of Pα5 significantly. The calibration standards, in order of increasing elution volume, were as follows: native BPTI; reduced, carboxymethylated BPTI; oxidized insulin B chain; PαPβ (Oas & Kim, 1988); $(PNPA)_3$; Ac-AKFERQHMDS. Pα5 was loaded at a concentration of 0.63 mM.

Results

Folding of Pα5 at Low Temperature. The CD spectrum of Pα5 at 0 °C, pH 7 (Figure 2), displays a weak but clear minimum at 222 nm. This minimum is severely reduced at 60 °C and shifts to higher wavelength in the presence of Gdn-HCl. These observations suggest that Pα5 has a small amount of structure at low temperature, which may be α-helical. The spectrum at 0 °C, 0 M Gdn-HCl does not display the two other characteristic α-helix bands; a minimum at 208 nm and a maximum near 192 nm [see review by Woody (1985)]. If only a small amount of α-helical structure is present, however, then contributions to the CD spectrum from the predominantly unfolded peptide are expected to mask these bands. Thus, the intense minimum at 205 nm is likely to result from "random coil" contributions [see review by Woody (1985)].

The temperature dependence of $[\theta]_{222}$ for Pα5 at various concentrations of denaturant (Gdn-HCl) is shown in Figure 3. In the absence of Gdn-HCl, a marked nonlinearity in the temperature dependence is observed below 30 °C, similar to that observed with the C-peptide analogue RN54, which was designated a "weak helix former" (Shoemaker et al., 1988). The melting curves for Pα5 are approximately linear both in the presence of denaturant and at high temperatures in the

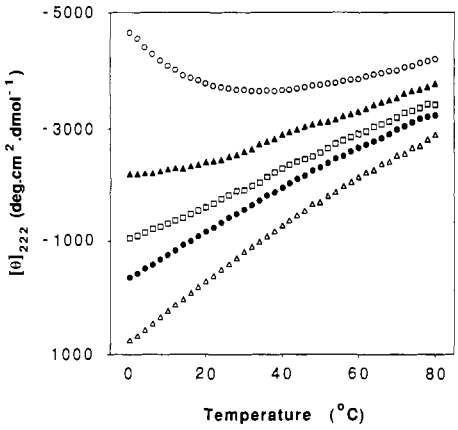

FIGURE 3: Temperature dependence of $[\theta]_{222}$ for Pα5 in 0.1 M NaCl/1 mM Na_2HPO_4, pH 7, at 0 M Gdn-HCl (○), 1 M Gdn-HCl, (▲), 2 M Gdn-HCl (□), 3 M Gdn-HCl (●), and 6 M Gdn-HCl (△).

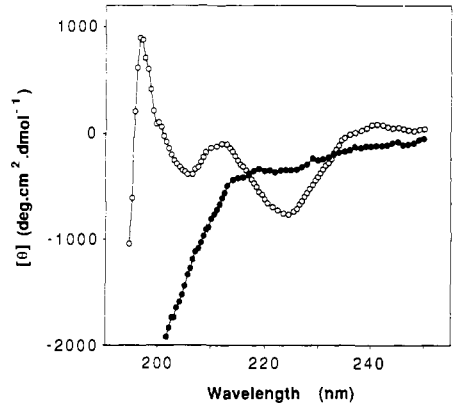

FIGURE 4: Difference CD spectra for Pα5 in 0.1 M NaCl/1 mM Na_2HPO_4, pH 7, for 0–15 °C (○) and 45–60 °C (●).

absence of Gdn-HCl. These results are indicative of structure formation at low temperature in the absence of Gdn-HCl. The nonlinear portion of the curve corresponds to the edge of what appears to be a broad, folding transition.

We have determined that Pα5 is monomeric under the conditions in which structure is observed. The apparent molecular weight of Pα5 as determined by Sephadex G-25 gel filtration at 4 °C, pH 6, is 1500 (theoretical M_r 1178). In addition, $[\theta]_{222}$ is independent (±2%) of Pα5 concentration (0.06–0.24 mM) at 0 °C, pH 7.

Evidence for α-Helical Structure in Pα5. In their studies of synthetic α-helical templates, T. P. Curran and D. S. Kemp used difference CD spectroscopy to monitor helix formation as residues were added progressively to a template (Curran, 1988). We have used a similar method to demonstrate that Pα5 forms an α-helix. Two temperatures were chosen (0 and 15 °C) in the nonlinear region of the melting curve (Figure 3). The rationale is that as the temperature is lowered, Pα5 becomes more structured and a difference spectrum should show characteristic CD bands arising from that structure. Pα5 also loses an equivalent amount of "random coil" structure, but since the ellipticity for the unstructured form is much less intense than that for an α-helix [see review by Woody (1985)] the contribution from the α-helix is expected to dominate in the difference spectrum. As a control, a difference spectrum at two high temperatures (45 and 60 °C), where the peptide is unfolded, was also obtained.

The resulting difference spectra are shown in Figure 4. The low-temperature spectrum is similar to that of an α-helix, as witnessed by the double minimum (224 and 206 nm) and single maximum (at 198 nm). The reference CD spectrum for an α-helix gives these band positions at 222, 208, and near

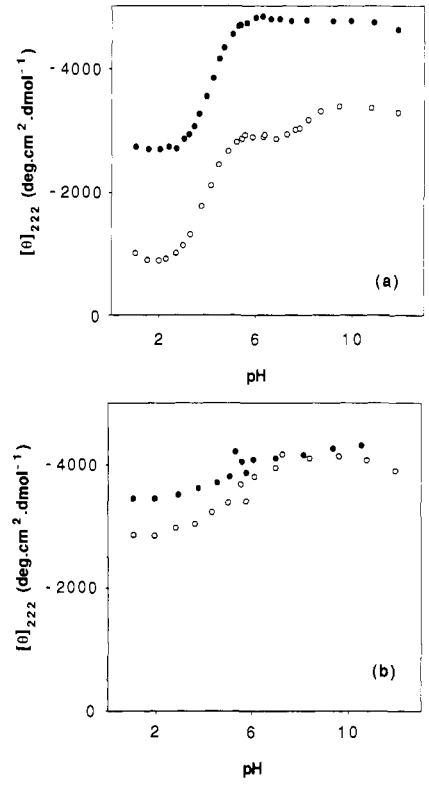

FIGURE 5: pH dependence of $[\theta]_{222}$ for Pα5 (●) and Pα7 (○) in 0.1 M NaCl, 10 mM Na_2HPO_4, and 10 mM NaOAc buffer at (a) 0 °C and (b) 60 °C.

192 nm, respectively [see review by Woody (1985)]. The control difference spectrum at high temperatures is clearly very different. Not only are there no minima above 210 nm, but also the spectrum shows no tendency to give a positive CD signal below 210 nm, as is observed in the low-temperature difference spectrum and in helical reference spectra. These results indicate that the structure of Pα5 at low temperature is α-helical, and we propose that this method of difference CD spectroscopy is a sensitive means for determining helical structure in peptides which show a small amount of folded structure at low temperatures.

Effect of Charges on Helix Stability. The helicity of Pα5 shows a marked pH dependence, which is consistent with predictions based on helix dipole and/or salt bridge considerations. Figure 5 shows the pH dependence of $[\theta]_{222}$ at 0 and 60 °C for Pα5 and Pα7 (which is identical in sequence with Pα5 but has a free N-terminus). At low temperature, helix content in Pα5 is maximal above pH 6. The observed titration with an apparent pK_a near 4.5 corresponds to deprotonation of one or more of the three acidic groups: Glu-49, Asp-50, and the α-carboxylate of Ala-58. NMR data (unpublished results) indicate that these three groups titrate in this pH range. The pH dependence is consistent with helix stabilization via electrostatic interactions between Glu-49, Asp-50, and the positive end of the helix dipole [cf. Shoemaker et al. (1987)]. The titration of Ala-58 may be expected to be helix destabilizing for the same reasoning; however, this residue may not be part of the α-helix, as the last helical residue in native BPTI is Gly-56 (Deisenhofer & Steigemann, 1975; Wlodawer et al., 1984, 1987). Alternatively, the observed helix stabilization may be due to salt bridge formation between Glu-49 and Arg-53 or Asp-50 and Arg-53; the latter type is observed in crystal form I of BPTI (Deisenhofer & Steigemann, 1975).

Pα7 shows a similar pH dependence of $[\theta]_{222}$ below pH 7. Above pH 9, however, further stabilization is observed as the N-terminus titrates. This observation is consistent with re-

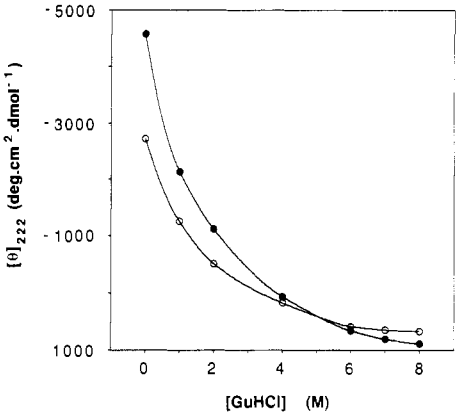

FIGURE 6: Gdn-HCl concentration dependence of $[\theta]_{222}$ for Pα5 (●) and Pα7 (○) in 0.1 M NaCl/1 mM Na_2HPO_4, pH 7, 3 °C.

moval of an unfavorable interaction between the positive end of the helix dipole and the positively charged N-terminus [cf. Shoemaker et al. (1987)].

The observed offset between the 0 °C titration curves for the two peptides probably results from an extra helical H bond that can be made in Pα5 by virtue of its additional C=O group in the acetylated N-terminus. At 60 °C, where the peptides are unfolded, neither peptide shows a strong pH dependence of $[\theta]_{222}$ (Figure 5b).

Estimate of Helix Content. We have used two methods to estimate the helical content of Pα5 at 0 °C, pH 7. The first method assumes that the base-line value for 0% helix is given by extrapolation to 0 °C of the linear portion of the temperature dependence for $[\theta]_{222}$ in the absence of Gdn-HCl (Figure 3). The value of $[\theta]_{222}$ so obtained is −2880 deg cm^2 dmol^{-1}, using the portion of the curve in the 60–80 °C region, which gives a correlation coefficient of 0.996. This value is changed by ~5% if only the portion between 70 and 80 °C is used, whereas inclusion of points lower than ~55 °C gives progressively worse correlation coefficients. The difference between the observed value of $[\theta]_{222}$ and the extrapolated base-line value at 0 °C is −1770 deg cm^2 dmol^{-1}.

Assuming that the helix in Pα5 (12 residues) is confined to the same 10 residues as in native BPTI (Deisenhofer & Steigemann, 1975; Wlodawer et al., 1984, 1987), this value is multiplied by 12/10 [cf. Bierzynski et al. (1982) and Kim and Baldwin (1984)]. Using a value for $[\theta]_{222}$ of −28 000 deg cm^2 dmol^{-1} for 100% helix content with a helix length of 10 residues (Chang et al., 1978) results in an estimated helical content for Pα5 at 0 °C, pH 7, of 8% ± 2%. The uncertainty in this estimate results primarily from inaccuracy in the ninhydrin concentration determination.

In their studies of C-peptide, Shoemaker et al. (1988) conclude that our first method underestimates the actual helix content in peptides. They argue that a more accurate base-line value for 0% helix is given by $[\theta]_{222}$ measured in concentrated solutions of Gdn-HCl. The argument is as follows (Shoemaker et al., 1988): (i) P-Peptide, which corresponds to residues 1–8 of C-peptide but does not form an α-helix, has a value of $[\theta]_{222}$ at 3 °C that is essentially independent of [Gdn-HCl]. (ii) The values of $[\theta]_{222}$ at 3 °C for strong, moderate, and weak helix-forming C-peptide variants all coalesce with each other and with that for P-peptide, at 8 M Gdn-HCl. (iii) Except in weak helix-forming C-peptide variants, the helix does not appear to be unfolded completely at high temperatures, so that base-line extrapolations such as we use above are likely to result in an underestimate of helix content.

The second method that we have used to estimate helix content in Pα5 follows that of Shoemaker et al. (1988). The dependences of $[\theta]_{222}$ in Pα5 and Pα7 on [Gdn-HCl] were measured at 3 °C, pH 7 (Figure 6). At high [Gdn-HCl], both peptides yield similar values of $[\theta]_{222}$ [cf. Figure 2a in Shoemaker et al. (1988)]. Assuming that the value at 8 M Gdn-HCl (+890 deg cm^2 dmol^{-1}) corresponds to the value for 0% helix content (Shoemaker et al., 1988), we obtain 23% ± 6% for the helix content of Pα5 at 0 °C, pH 7. We regard our first estimate (8%) as a lower limit and our second estimate (23%) as an upper limit of the helix content in Pα5.

Discussion

Our results provide a third example of a native sequence in a short peptide which shows partial α-helical structure in aqueous solution at low temperatures. The C-terminal α-helix of BPTI is apparently important for the stability of early folding intermediates in the oxidative folding of BPTI (Creighton, 1978). When BPTI is cleaved at Met-52 with cyanogen bromide, the reduced fragment cannot refold and produces a random mixture of disulfide bonds under folding conditions. This is in contrast to intact BPTI, where a distinctly nonrandom mixture of one and two-disulfide intermediates is obtained (Creighton, 1977). Although the 1–52 BPTI fragment is missing a crucial cysteine residue (Cys-55), this alone cannot account for the lack of the two predominant one-disulfide intermediates observed in the folding of native BPTI ([30–51] and [5–30]), neither of which involves Cys-55.

Recently, a peptide model of the 30–51 one-disulfide intermediate of BPTI has been made and is ~90% folded at 0 °C (Oas & Kim, 1988). The observed helicity of Pα5 is particularly interesting in light of this since Pα5 comprises approximately one-third of the residues in the peptide model (PαPβ). This will enable us to make amino acid changes that alter helix content in Pα5 and then to observe the effect of these changes on the stability of PαPβ as tertiary structure is introduced to the system. In this way, we hope to build a stepwise picture of the intermediates involved at increasing hierarchical levels of structure and at different stages in the folding process.

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References

Bierzynski, A., Kim, P. S., & Baldwin, R. L. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 2470–2474.

Brown, J. E., & Klee, W. A. (1971) *Biochemistry 10*, 470–476.

Chang, T. C., Wu, C.-S., & Yang, J. T. (1978) *Anal. Biochem. 91*, 13–31.

Creighton, T. E. (1977) *J. Mol. Biol. 113*, 275–293.

Creighton, T. E. (1978) *J. Mol. Biol. 119*, 507–518.

Curran, T. P. (1988) Ph.D. Thesis, Department of Chemistry, Massachusetts Institute of Technology.

Deisenhofer, J., & Steigemann, W. (1975) *Acta Crystallogr., Sect. B 31*, 238–250.

Dyson, H. J., Cross, K. J., Houghten, R. A., Wilson, I. A., Wright, P. E., & Lerner, R. A. (1985) *Nature 318*, 480–483.

Dyson, H. J., Rance, M., Houghten, R. A., Wright, P. E., & Lerner, R. A. (1988) *J. Mol. Biol. 201*, 201–217.

Gilbert, W. (1978) *Nature 271*, 501.
Gilbert, W. (1985) *Science 228*, 823–824.
Kent, S. B. H. (1988) *Annu. Rev. Biochem. 57*, 957–989.
Kim, P. S., & Baldwin, R. L. (1982) *Annu. Rev. Biochem. 51*, 459–489.
Kim, P. S., & Baldwin, R. L. (1984) *Nature 307*, 329–334.
Marqusee, S., & Baldwin, R. L. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 8898–8902.
Oas, T. G., & Kim, P. S. (1988) *Nature 336*, 42–48.
Ptitsyn, O. B. (1987) *J. Protein Chem. 6*, 273–293.
Richardson, J. S. (1985) *Methods Enzymol. 115*, 359–380.
Rosen, H. (1957) *Arch. Biochem. Biophys. 67*, 10–15.
Shoemaker, K. R., Kim, P. S., York, E. J., Stewart, J. M., & Baldwin, R. L. (1987) *Nature 326*, 563–567.
Shoemaker, K. R., Fairman, R., York, E. J., Stewart, J. M., & Baldwin, R. L. (1988) in *Proceedings of the 10th American Peptide Symposium* (Marshall, G. R., Ed.) pp 15–20, ESCOM, Leiden.
Tam, J. P., Heath, W. F., & Merrifield, R. B. (1986) *J. Am. Chem. Soc. 108*, 5242–5252.
Woody, R. W. (1985) in *The Peptides* (Udenfriend, S., & Meienhofer, J., Eds.) Vol. 7, pp 15–114, Academic Press, New York.
Wlodawer, A., Walter, J., Huber, R., & Sjölin, L. (1984) *J. Mol. Biol. 180*, 301–329.
Wlodawer, A., Nachman, J., Gilliland, G. L., Gallagher, W., & Woodward, C. (1987) *J. Mol. Biol. 198*, 469–480.
Yajima, H., & Fujii, N. (1983) in *The Peptides* (Udenfriend, S., & Meienhofer, J., Eds.) Vol. 5, pp 65–109, Academic Press, New York.

Kinetic Properties of the Na^+/H^+ Antiport of Heart Mitochondria†

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ABSTRACT: The fluorescence of 2′,7′-bis(carboxyethyl)-5(6)-carboxyfluorescein (BCECF) has been used to follow the Na^+/H^+ antiport activity of isolated heart mitochondria as a Na^+-dependent extrusion of matrix H^+. The antiport activity measured in this way shows a hyperbolic dependence on external Na^+ or Li^+ concentration when the external pH (pH_o) is 7.2 or higher. The apparent K_m for Na^+ decreases with increasing pH_o to a limit of 4.6 mM. The K_i for external H^+ as a competitive inhibitor of Na^+/H^+ antiport averages 3.0 nM (pH_o 8.6). The V_{max} at 24 °C is 160 ng ion of H^+ min^{-1} (mg of protein)$^{-1}$ and does not vary with pH_o. Li^+ reacts with the antiporter with higher affinity, but much lower V_{max}, and is a competitive inhibitor of Na^+/H^+ antiport. The rate of Na^+/H^+ antiport is optimal when the pH_i is near 7.2. When pH_o is maintained constant, Na^+-dependent extrusion of matrix H^+ shows a hyperbolic dependence on $[H^+]_i$ with an apparent K_m corresponding to a pH_i of 6.8. The Na^+/H^+ antiport is inhibited by benzamil and by 5-N-substituted amiloride analogues with I_{50} values in the range from 50 to 100 μM. The pH profile for this inhibition seems consistent with the availability of a matrix binding site for the amiloride analogues. The mitochondrial Na^+/H^+ antiport resembles the antiport found in the plasma membrane of mammalian cells in that Na^+, Li^+, and external H^+ appear to compete for a common external binding site and both exchanges are inhibited by amiloride analogues. However, there are significant differences in the sensitivity of the two antiports to these inhibitors, and the mitochondrial exchanger appears to operate in a more alkaline region than the plasmalemmal component. The increased affinity of the antiport for Na^+ with increasing pH is in line with the putative role of this exchanger as a device for extruding Na^+ from the alkaline matrix of respiring mitochondria.

Isolated heart mitochondria can be loaded with the fluorescent pH indicator 2′,7′-bis(carboxyethyl)-5(6)-carboxyfluorescein (BCECF)[1] by procedures quite analogous to those used with intact cells (Davis et al., 1987a; Jung et al., 1988). Studies from this laboratory have established that changes in matrix pH (pH_i) reported by BCECF fluorescence are in close agreement with values obtained by the distribution of acetate or DMO (Jung et al., 1988) and that these procedures provide a convenient and continuous readout of pH_i with changing metabolic conditions (Davis et al., 1987a). Estimation of pH_i by BCECF fluorescence appears to be free of the binding artifacts that affect the distribution of weak base pH probes, such as methylamine (Jung et al., 1988), and with a pK_a near 7.0 (Rink et al., 1982), BCECF can effectively report pH_i over a range from 6.0 to 8.0.

In the present study, the fluorescence of BCECF is used to monitor the changes in pH_i that result from monovalent cation/H^+ antiport reactions across the inner membrane of the mitochondrion. Mitochondria appear to contain both an

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[1] Abbreviations: SMP, submitochondrial particle(s); BCECF, 2′,7′-bis(carboxyethyl)-5(6)-carboxyfluorescein; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; pH_o, pH of mitochondrial suspending medium; pH_i, pH of matrix compartment as reported by BCECF fluorescence; TES, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid; TEA^+, tetraethylammonium ion; DMO, 5,5′-dimethyl-2,4-oxazolidinedione; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid.

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