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Refolding and Assembly of Penicillin Acylase, an Enzyme Composed of Two Polypeptide Chains That Result from Proteolytic Activation†

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ABSTRACT: The in vitro folding and assembly of penicillin acylase (EC 3.5.1.11) (PA) to active enzyme has been studied. PA is a large bacterial protein (M_r = 86 000) comprising two peptides, α and β, produced by proteolytic processing and activation of a 92-kDa precursor. Proteins that result from proteolytic processing are characteristically difficult if not impossible to refold. Different factors that affect folding and assembly of PA, including pH, ionic strength, and temperature, have been studied. Yields of 60% can be obtained, based on recovery of enzyme activity, together with another 20% of folded and associated monomer with conformation closely similar to that of the active enzyme but with the active site not formed. Evidence is presented for in vitro assembly proceeding via initial folding of the N-terminal α-peptide with subsequent collapse of the transiently folded β-chain on to the surface of the former. A slow process of rearrangement follows association in vitro. Competition experiments support the proposal that the linker endopeptide in the precursor serves to increase the probability of productive collision between folded α- and β-peptides. The effect of raised temperature is to interfere with the folding of the α-peptide, thus preventing proper folding of the precursor. This finding accounts for the basis of the temperature regulation of PA production in vivo.

Proteins that derive from precursors by proteolytic processing and activation do not in general unfold reversibly (Anfinsen, 1967). Cathepsin D (Lah et al., 1984) and pepsin (Ahmed & McPhie, 1978) each originate from precursors that will fold in vitro as well as in vivo, but the enzymes that have lost an N-terminal activation peptide are metastable. Once unfolded, it has not been possible to regain any enzyme activity for either enzyme. Chymotrypsin, like insulin, consists of peptide chains held together by disulfide bonds as well as noncovalent interactions. Incubation with protein disulfide isomerase leads to disulfide band exchange and ultimately inactivation and aggregation (Givol et al., 1965). These proteins, unlike their precursors, are therefore not in their lowest accessible free energy states. One of the tenets of the thermodynamic hypothesis is that the information required to specify the folding of a globular protein is contained in the primary sequence and the lack of reversibility has been ascribed tentatively to loss of information residing in the amino acid sequence (Anfinsen, 1967).

Penicillin acylase (EC 3.5.1.11) is a large (M_r = 86 000) monomeric enzyme unique among bacterial enzymes in being activated by proteolytic processing of a single polypeptide

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chain, M_r = 95000 (Böck et al., 1983a,b). The two peptides, α (M_r = 23800) and β (M_r = 62200) that comprise the enzyme are strongly associated through noncovalent interactions, and denaturation causes unfolding and dissociation of the protein in a highly cooperative manner (Lindsay & Pain, 1990). The N-terminal peptide—but not the β-peptide—will fold independently to a compact globular conformation with secondary and tertiary structure, exhibiting on its surface a nonpolar ANS-binding patch that is, however, not exposed to solvent in the active enzyme. It was proposed that the precursor folds sequentially, the C-terminal β-peptide portion folding and binding to the already folded α-peptide portion of the chain. An investigation of the folding and association of the two peptides should throw light on the folding of the large precursor chain that is intimately connected with its translocation and processing (Sizmann et al., 1990).

Such an investigation could also be important for biotechnology. The enzyme, which is important in the semisynthesis of penicillins, cannot be produced at high levels by standard recombinant DNA techniques owing to the necessary involvement of the translocation mechanism. It has, however, been possible to express genes corresponding to the α- and β-peptides and, at high levels, these lead to readily purified inclusion bodies (D. Sizmann, personal communication). If these could be renatured and induced to associate it would provide an alternative route to large quantities of wild-type and mutant penicillin acylases.

We describe the successful refolding and assembly of the two peptides to give active enzyme in high yield. Insights into the pathway of folding of this large protein have been obtained that allow the temperature-regulated expression of PA in vivo to be explained in terms of protein folding rather than gene regulation.

Experimental Procedures

Materials

Penicillin G acylase (PA)[1] from *Escherichia coli* strain ATCC 11105 was supplied in 95% pure form as an ammonium sulfate precipitate by Boehringer Mannheim. Ultrapure urea (enzyme grade) was from Gibco BRL. Filters (0.45 μm, 47-mm diameter) for buffers for fast protein liquid chromatography (FPLC) were from Gelman Sciences Inc., and 0.22-μm (13-mm diameter) filters for spectroscopic clarification were from Millipore. Phenylmethanesulfonyl fluoride (PMSF) and 6-nitro-3-(phenylacetamido)benzoic acid (NIPAB) were obtained from Sigma. Protein stock solutions were clarified by centrifugation at 10000 rpm at 4 °C for 15 min prior to use.

Unless otherwise stated, all the procedures described were carried out using the "borate buffer" system (Gomori, 1955). This contains acetate, phosphate, and borate ions and varies in ionic strength from 0.28 mol·L^{-1} at pH 8.43 to 0.13 mol·L^{-1} at pH 4.43.

Methods

"*Unfolded PA*" was generated by dissolving PA (1–3 mg/mL) in 8.8 M urea/50 mM phosphate buffer, pH 7.5, and dialyzing against 100 mL of the same buffer for 24 h at 22 °C. α- and β-peptides were prepared by separation on a Pharmacia Superose 12 HR10/30 gel-exclusion FPLC column equilibrated with the above buffer and eluted at 20 mL/h.

Assembly of PA was carried out by dilution of unfolded PA stock (2.5×10^{-6} mol·L^{-1}) into buffers of pH 4.3–8.4 to a final protein concentration of 5.5×10^{-8} mol·L^{-1}. The diluted PA solution was incubated at 22 °C for 4 h. Assembly was also carried out from unfolded α- and β-peptides (1.3×10^{-5} and 7.4×10^{-6} mol·L^{-1}, respectively) in 8.8 M urea.

Assembly with molar excess α-peptide was carried out by using unfolded α-peptide refolded by dialysis of a 2.0×10^{-5} mol·L^{-1} solution against refolding buffer, pH 6.5, at 4 °C for 24 h. Unfolded β-peptide (4.2×10^{-6} mol·L^{-1}) was diluted into varying concentrations of refolded α-peptide in pH 6.5 buffer at 4 °C and incubated for 4 h. The final concentration of β-peptide was 6.4×10^{-8} mol·L^{-1}. The solutions were equilibrated at 22 °C for 30 min prior to determination of PA activity.

The recovery of refolded PA was determined from its enzymic activity at the refolding pH and 22 °C as a proportion of the activity of solutions of native PA at equimolar concentrations and at the same pH.

Assembly by Dialysis. Unfolded PA (0.84–2.3 mg) in a volume of 0.7–1.25 mL was dialyzed against 2.5–5.0 L of borate buffer, pH 6.5, at 4 °C for 24 h. Solubilized, refolded PA was separated from aggregated PA by centrifugation at 10000 rpm for 15 min at 4 °C. The pellet containing 0.09–0.28 mg of PA was resolubilized in 1 mL of 8.6 M urea buffer, pH 6.5, and incubated at 22 °C for 1 h prior to dialysis against buffer at 4 °C for 24 h. Solubilized, refolded PA was separated from aggregated PA by centrifugation as above.

Elution volumes were determined by chromatography of 0.02–0.29 mg of PA on a Pharmacia Superose 12 HR10/30 gel-exclusion FPLC column equilibrated in the appropriate buffer at 20 °C.

Circular dichroism spectra were measured by using a Jobin-Yvon dichrographe IV, standardized with epiandrosterone (Jobin-Yvon, Longjumeau, France). A thermostated 10-mm path length cell was employed. Ellipticity at 25 °C was expressed as mean residue weight ellipticity ($[\Theta]_{mrw}$ deg·cm^2·dmol^{-1}).

Fluorescence emission was measured at 25 °C by using a Perkin Elmer MPF-3L fluorescence spectrophotometer with thermostated cell. Fluorescence emission was determined with an excitation wavelength of 280 nm.

Protein concentration of PA was measured by absorbance at 280 nm by using $A^{1mg\cdot mL^{-1}}_{280}$ = 2.22 (M_r = 86031).

Enzyme activity was measured by hydrolysis of the chromogenic substrate NIPAB at a concentration of 1.1×10^{-4} mol·L^{-1} in pH 6.5 buffer at 22 °C (Kutzbach & Rauenbusch, 1974). Absorbance was measured at 405 nm as a function of time with PA concentrations of approximately 6.5×10^{-8} mol·L^{-1}.

Active-Site Titration. The enzymic activity of PA was irreversibly inhibited by titration with PMSF (Kutzbach & Rauenbusch, 1974). Solutions of enzyme (6.30×10^{-8} mol·L^{-1}) were incubated with 0–8.8×10^{-8} mol·L^{-1} PMSF at 22 °C for 30 min in pH 6.5 buffer. The residual activity was determined as above by the addition of NIPAB to a final concentration of 1.1×10^{-4} mol·L^{-1}.

Results

Unfolded and disassociated α- and β-peptides were obtained by incubation of purified PA with 8.8 M urea at pH 7.5 (see Methods) and will be referred to as unfolded PA. This was characterized by FPLC gel-exclusion chromatography in the same solvent medium, which showed two elution bands corresponding to fully unfolded peptides of the appropriate molecular weight. No higher molecular weight material was

[1] Abbreviations: CD, circular dichroism; FPLC, fast protein liquid chromatography; NIPAB, 6-nitro-3-(phenylacetamido)benzoic acid; PA, penicillin acylase (penicillin aminohydrolase) (EC 3.5.1.11); PMSF, phenylmethanesulfonyl fluoride.

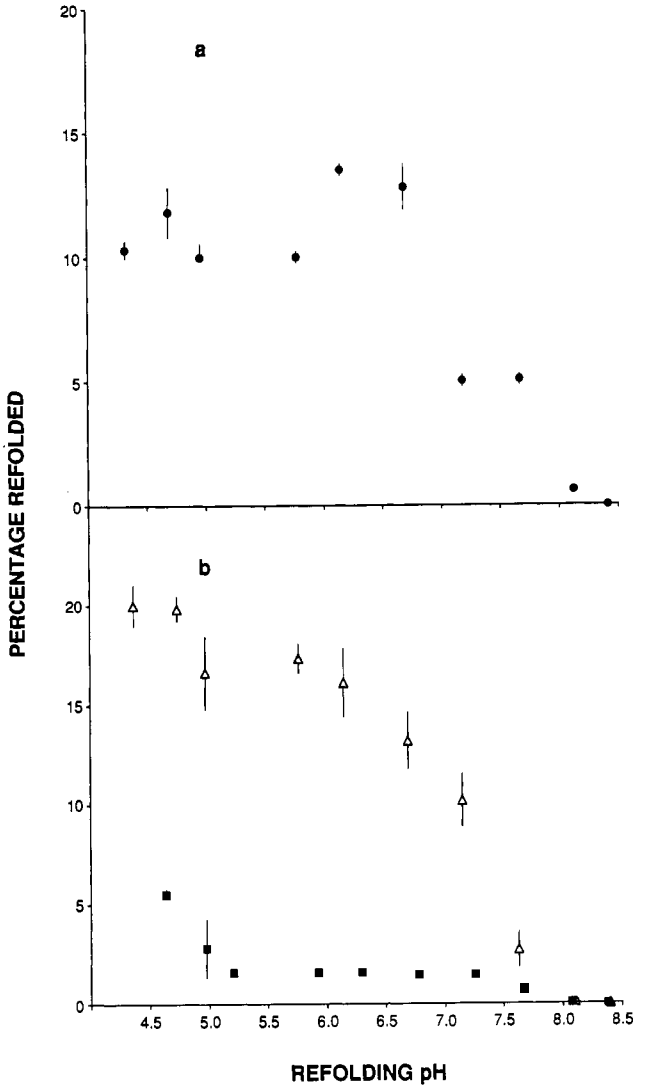


FIGURE 1: Assembly of penicillin acylase as a function of pH. (a) Unfolded PA in 8.6 M urea was diluted into borate buffer (see Methods) at 22 °C (●). Final concentration of PA 5.6 × 10^{-7} mol·L^{-1}; final concentration of urea 0.19 mol·L^{-1}. (b) Equimolar amounts of unfolded β-peptide were added to α-peptide incubated in refolding buffer (borate buffer, pH 6.5) (△) and unfolded α-peptide added to β-peptide (again in equimolar amounts) incubated in refolding buffer (■). Final concentrations of PA (based on total α- and β-peptides) were 4.4 × 10^{-7} and 2.0 × 10^{-7} mol·L^{-1}, respectively; final concentrations of urea were 0.33 and 0.47 mol·L^{-1}, respectively. Recovery of PA activity was monitored by using NIPAB as substrate at the pH of assembly and was expressed as a percentage of the activity of the same concentration of native PA at the same pH.

found. For some experiments the peptides were separated by preparative FPLC.

Dependence of Folding on pH. Refolding was initiated by dilution of unfolded PA into buffer as described under Methods to give final solvent conditions under which the native enzyme is stable and active. Similar experiments carried out as a function of pH (Figure 1a) showed that active enzyme was formed with yields of more than 10% between pH 4 and 7. Experiments with separated α- and β-peptides showed that the recovery of activity depended on the order of mixing. Addition of unfolded α-peptide to β-peptide previously equilibrated under refolding conditions led to zero recovery of activity, whereas the addition of unfolded β-peptide to already refolded α-peptide led to improved yields of up to 20% at the lower pH range (Figure 1b).

α-Peptide can be refolded with close to 100% efficiency (Lindsay & Pain, 1990). In order to locate the determining

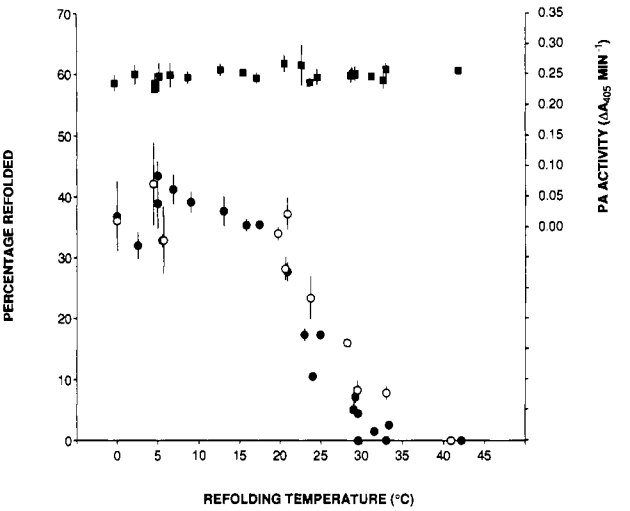


FIGURE 2: Assembly of penicillin acylase as a function of temperature. Unfolded PA was diluted into borate buffer, pH 6.5, at the temperatures indicated (●). Final concentration of total PA protein was 6.4 × 10^{-8} mol·L^{-1}; final concentration of urea was 0.12 mol·L^{-1}. Native PA was similarly incubated in borate buffer, pH 6.5 (■). Solutions and controls were maintained at the temperature for 4 h and then equilibrated at 22 °C for 30 min prior to measurement of activity against NIPAB. Assembly was repeated as above in the presence of 1 M NaCl (○).

factor in pH-dependent folding, the α-peptide was studied separately. When it was refolded over the same pH range as in Figure 1, the degree of refolding was found to be independent of pH as determined by fluoresence intensity at 320 nm and by the wavelength of maximum fluorescence emission (334 nm), neither of which quantities varied between pH 4.5 and 8.5. Association of α- and β-peptides is largely hydrophobic in nature (Lindsay & Pain, 1990), suggesting that the pH dependence of assembly arises from a pH dependence of folding of the β-peptide rather than of association.

Dependence of Folding on Temperature. The assembly of active enzyme from unfolded PA was studied as a function of temperature at pH 6.5 (Figure 2). Native PA is seen to be stable over the range 0–45 °C. Yields of assembly, however, approach 40% between 0 and 17 °C but fall off sharply to zero between 20 and 30 °C.

To locate the source of this temperature dependence, α-peptide was refolded over the same range of temperatures and its fluorescence measured at each temperature to avoid problems of reversibility. The wavelength of maximum emission was independent of temperature, showing that substantial folding of the α-peptide had occurred. The intensity of fluorescence decreased sharply above 20 °C (Figure 3) accompanied by a similar sharp increase in the intensity of the Rayleigh scattering peak (data not shown), indicating that the α-peptide associates during or after folding at higher temperatures.

Dependence of Folding on Ionic Strength. The effect of ionic strength on the assembly process was investigated in 50 mM phosphate buffer, pH 6.5, at 22 °C by adding sodium chloride from 0 to 5 mol·L^{-1}. The activity of PA itself was found to be markedly sensitive to ionic strength (Figure 4a), although the near-UV CD of PA is independent of ionic strength in the range 0.1–2.5 mol·L^{-1} NaCl (data not shown). No activity is recovered on refolding at or above 2.5 M NaCl. Calculating the yield of active, reassembled PA as a proportion of the activity of native PA at the relevant ionic strength, an optimum yield is obtained between 0.5 and 1.5 mol·L^{-1} NaCl (Figure 4b). The possibility of using this result to extend the range of temperature over which the enzyme could be assem-

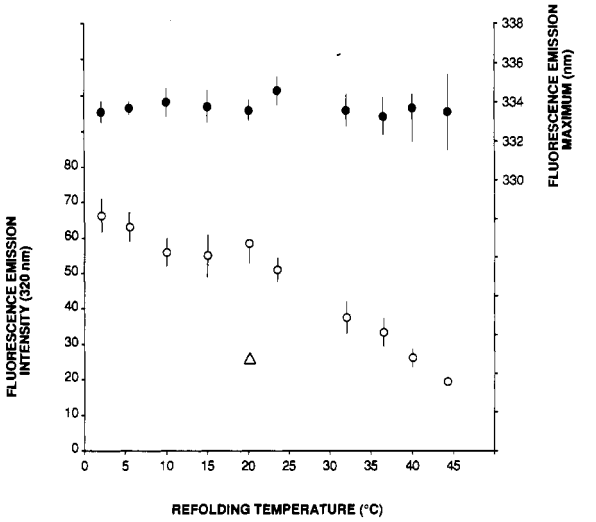


FIGURE 3: Refolding of α-peptide as a function of temperature. Unfolded α-peptide (in 8.6 M urea) was refolded by dilution into borate buffer, pH 6.5, at temperatures from 2.1 to 44.3 °C and incubated for 3 h. Final concentration of α-peptide was 2.6×10^{-6} mol·L^{-1}; final concentration of urea was 0.33 mol·L^{-1}. The conformation of the α-peptide was monitored by fluorescence at the temperature employed for the incubation by using fluorescence emission maximum (●) and emission intensity (○). Fluorescence emission intensity of unfolded α-peptide is also shown (△). Fluorescence was excited at 280 nm. The data are presented as means of three determinations ± standard deviation.

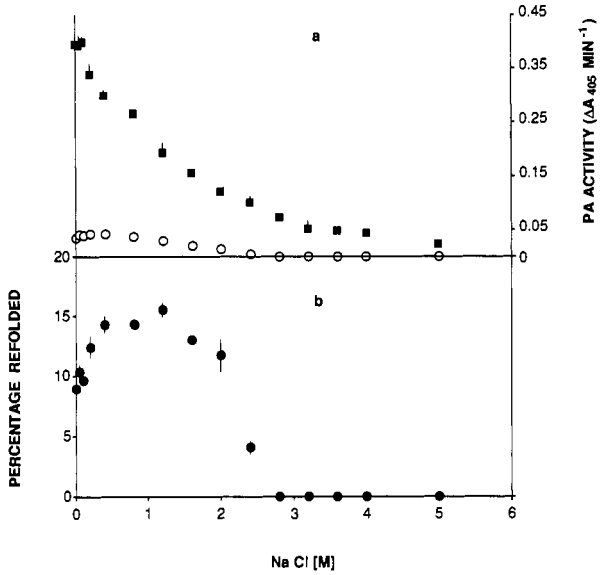


FIGURE 4: Assembly of penicillin acylase as a function of ionic strength. (a) Unfolded PA was diluted into 50 mM phosphate buffer, pH 6.5, containing different concentrations of NaCl and incubated for 4 h at 22 °C (○); the activity of native PA similarly incubated in the same buffers was measured (■). (b) From the data in (a), the percentage recovery of PA activity is calculated (●). Final concentration of total PA protein was 5.5×10^{-8} mol·L^{-1}; final concentration of urea was 0.19 mol·L^{-1}.

bled was explored by studying the effect of temperature on assembly in the presence of 1.0 mol·L^{-1} NaCl. The results suggest a shift of the assembly curve to higher temperatures by not more than 5 °C. There was in addition no effect on the yield at lower temperatures (Figure 2).

Effect of Molar Ratio of Peptides on Assembly. It has previously been suggested (Lindsay & Pain, 1990) that the β-peptide part of the PA precursor folds and associates with the hydrophobic patch on the already folded N-terminal α-peptide and that the efficiency of this process derives from the

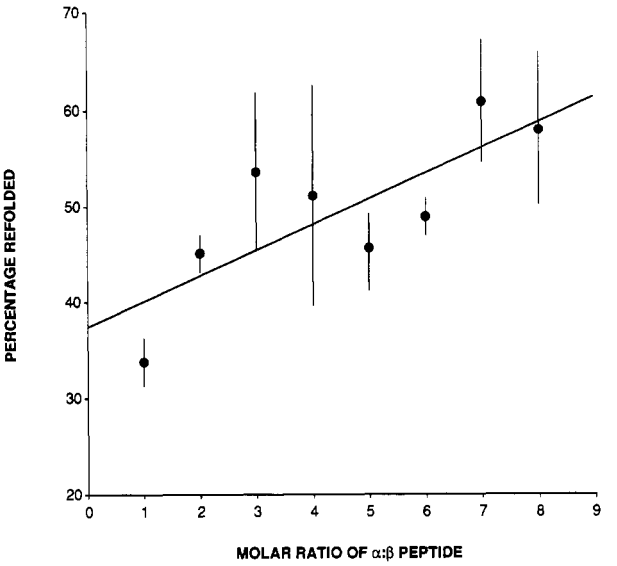


FIGURE 5: Recovery of penicillin acylase as a function of α-peptide concentration. Unfolded β-peptide was diluted into varying concentrations of refolded α-peptide (see Methods) in borate buffer, pH 6.5, at 5.7 °C. The percentage recovery of PA activity based on the total concentration of β-peptide is plotted against the molar ratio of α-peptide:β-peptide at constant final concentration of the latter (6.4×10^{-8} mol·L^{-1}). Final concentration of urea was 0.13 mol·L^{-1}. The vertical bars indicate standard deviations for means of three determinations.

linking peptide that favors α to β interaction at the expense of β to β interaction. This hypothesis, already supported by the results in Figure 1, was further tested by increasing the molar proportion of α- to β-peptide. Unfolded β-peptide was added to varying concentrations of previously refolded α-peptide at pH 6.5. It was found that the yield of PA activity, based on the amount of β-peptide used, increases from 40% to 60% as the molar ratio of α to β is increased from 1:1 to 8:1 (Figure 5).

Kinetics of Folding and Assembly. The kinetics of recovery of PA activity were examined at a molar ratio of peptides of 1:1. Activity is regained sufficiently slowly for aliquots of refolding solution to be assayed, showing that approximately 90 min ($t_{1/2} \sim 15$ min) is required to achieve maximum activity at pH 6.5 and 22 °C at a protein concentration of 6.4×10^{-8} mol·L^{-1} and a final urea concentration of 0.14 mol·L^{-1}. When assembly was initiated by mixing and dilution, the final concentration of urea was finite (see figure legends) but the recovery of active PA was shown to be independent of urea concentration between 0 and 0.7 mol·L^{-1}.

Characterization and Optimization of Yield of Reassembled PA. In the above experiments refolding was initiated by rapid dilution of unfolded peptides in urea to refolding conditions at protein concentrations in the range of 10^{-7} mol·L^{-1} (6 μg·mL^{-1} β-peptide). Low protein concentrations were found to be essential to avoid reduced recovery of activity resulting from aggregation. Further experiments were performed in which denaturant was removed by dialysis. Unfolded PA in 8.8 M urea was dialyzed for 24 h against a 5×10^3-fold volume excess of borate buffer, pH 6.5, at 4 °C and at protein concentrations of 0.84–2.30 mg·mL^{-1}. Removal of aggregate by centrifugation at 10000 rpm yielded a supernatant containing PA enzyme activity at a level of 49% (averaged over six experiments) of that of the starting enzyme. The inactive aggregate was redissolved in 8.6 M urea and then redialyzed as above. The supernatant from this second renaturation contained a further 9% of the original PA activity, making a total recovery of 58% of the enzyme activity. No attempt was made

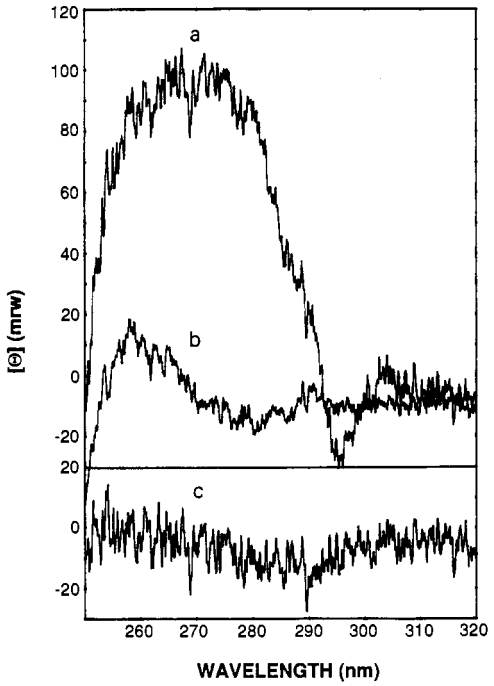


FIGURE 6: Circular dichroism spectrum of refolded and assembled penicillin acylase. Near-UV CD spectra of (a) refolded PA and (b) unfolded PA, and (c) difference spectrum of native PA against refolded PA. Refolded and native PA were in borate buffer, pH 6.5, at 25 °C at protein concentrations of 1.26×10^{-6} and 3.50×10^{-6} mol·L^{-1}, respectively; unfolded PA (2.39×10^{-6} mol·L^{-1}) was in buffer with 8.6 M urea.

Table I: Characteristics of Assembled Penicillin Acylase[a]

	fluorescence emission, max (nm)	fluorescence intensity at 320 nm[b] (%)	A_{280}/A_{250}	sp act.[b,c] (%)
native PA	334.0 ± 0.4	100	2.97 ± 0.02	100
unfolded PA	346.9 ± 0.7	46.4 ± 2.0	2.67 ± 0.07	0
refolded PA	334.2 ± 0.1	99.2 ± 0.7	2.86 ± 0.13	72.5

[a] Data are mean values of three determinations ± SD. [b] Calculated relative to value for native PA. [c] Based on absorbance at 280 nm.

to optimize this second stage, and higher yield should be attainable.

The recovery of PA activity in high yields and at high protein concentrations enabled the product to be characterized further. Protein in the supernatant from a single dialysis cycle as described above exhibited a single elution peak on gel-permeation chromatography with FPLC Superose-12. The elution volume, V_e = 13.47 ± 0.09 mL, corresponds to that of native PA, V_e = 13.47 ± 0.07 mL. No higher or lower molecular weight material was seen, indicating that the insoluble aggregate is formed from both α- and β-peptides, associated but not folded to the native state.

The near-UV CD spectrum is a sensitive fingerprint of tertiary interactions. The spectrum of reassembled PA shows a strong band of ellipticity in the aromatic region, indicating well-folded tertiary structure, and is indistinguishable from that of native PA as demonstrated by the CD difference spectrum (Figure 6).

The fluorescence emission maximum for PA is red-shifted on unfolding and the intensity of fluorescence at 320 nm is correspondingly reduced (Lindsay & Pain, 1990). Both parameters regain their native values in the reassembled protein (Table I).

Despite the evidence for the regain of native structure, the specific activity of the reassembled protein, based on UV absorbance, was only 72.5% that of native PA (Table I). To ascertain whether this represents homogeneous protein with a reduced enzymatic activity or native PA mixed with inactive protein, active-site titration experiments were carried out. PA is known to be inactivated by phenylmethanesulfonyl fluoride (PMSF) on a 1:1 (mol/mol) basis (Kutzbach & Rauenbusch, 1974). The activity of reassembled PA was fully titrated at a PMSF:protein molar ratio of 0.70:1 compared to 0.94:1 for the starting enzyme (Figure 7), while the slope of the titration curve remains constant. Thus 74% of the reassembled protein sample contains a specifically reactive serine, providing strong evidence for the 72.5% recovery of activity being accounted for by native, fully active enzyme.

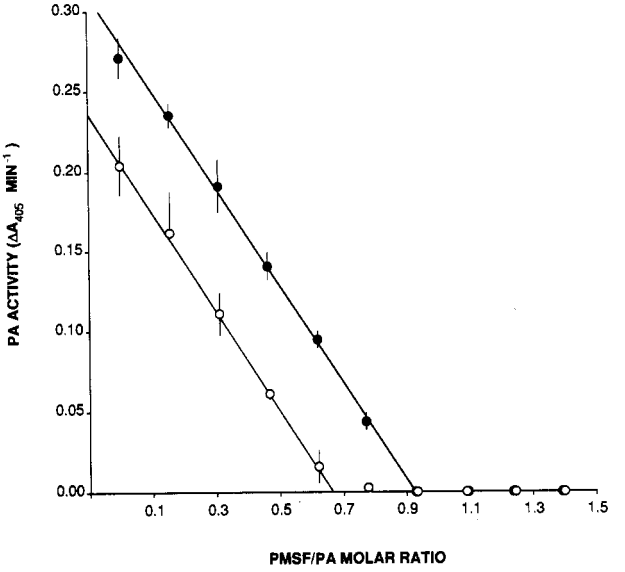


FIGURE 7: Active-site titration of refolded penicillin acylase with PMSF. The activity of native (●) and refolded (○) PA at concentrations of 6.4×10^{-8} mol·L^{-1} was measured after incubation with PMSF at 22 °C for 30 min in pH 6.5 buffer (see Methods).

The absorbance peak to trough ratio, A_{280}/A_{250}, is a sensitive measure of turbidity and hence of aggregation. The value for the refolded protein is not significantly lower than that for the native enzyme. The elution volumes of native and refolded proteins on gel-exclusion chromatography were found to be identical, both exhibiting elution peaks with sharp leading edges. No significant trend in specific activity in samples taken across the elution peak was obtained. It must be concluded therefore that the product of assembly contains 25% of folded but inactive PA with essentially the same Stokes' radius and spectroscopic properties as those for the native enzyme.

Discussion

Assembly of Active PA. The possibility of reassembly of PA in vitro from the α- and β-peptides was raised by experiments of Daumy et al. (1985). Activity was recovered by removing denaturant from PA (*Proteus rettgeri* and *E. coli*) dissolved in 6 M urea. Only 12% activity was recovered from previously separated α- and β-peptides. The present experiments show that the two constituent peptides of PA can be mixed, folded, and assembled to give active enzyme in more than 50% yield at protein concentrations in the 2 mg·mL^{-1} range. This yield can be significantly increased by recycling the aggregated side product. The active product has been shown to be identical with the native enzyme by spectroscopic techniques. The specific activity of the soluble protein separated from the gross aggregate by centrifugation is, however, approximately 75% of that of native PA. The results of PMSF

titration lead to the conclusion that the renatured protein contains the same proportion of active catalytic sites and therefore that 25% of the protein is nonfunctional. The possibility that this could be small aggregated protein was explored but it was concluded that the product is monomeric but inactive. The conformation must be closely similar to that of native PA to account for the circular dichroism and fluorescence results.

Studies of the refolding of rather smaller, single-polypeptide proteins have led to the general conclusion that refolding and conformation are tightly linked. This and the conformational similarity of the two PA refolding products suggest that the lack of activity may be attributed to a misorientation of the associated peptides, an argument consistent with the evidence that activity involves both peptides (Daumy et al., 1985).

The ability to refold and reassemble PA from the unfolded peptides with high yield affords a useful method for preparing the wild-type and modified enzymes by expressing the separately cloned genes for each peptide at high levels as stable inclusion bodies.

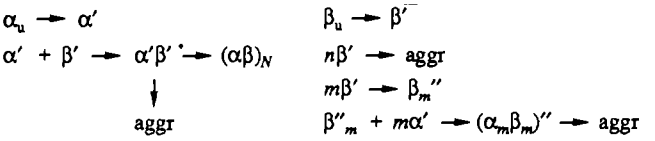
Mechanism of Assembly. Previous work on the refolding of PA (Lindsay & Pain, 1990) involving the use of urea gradient gel electrophoresis showed that β-peptide aggregates strongly but that α-peptide refolds reversibly. In this technique the α- and β-peptides, applied to the gel together in the same denaturing solution, are separated from each other as they migrate into lower urea concentrations. In the present experiments, the peptides coexist in solution throughout the change of solvent conditions. The fact that no free α-peptide is found after renaturation shows that, under these conditions, association of α- and β-peptides occurs in the formation of both active PA and inactive protein.

The demonstrated ability of the α-peptide to fold independently (Lindsay & Pain, 1990) coupled with the high yield of active PA enzyme indicates that the two peptides each contain the information required for them to be termed folding domains. The strong tendency of the β-peptide for rapid self-aggregation (Lindsay & Pain, 1990) to a state that will not combine with α-peptide to give active PA demonstrates the requirement for α- and β-peptides to combine at an early stage in the folding and assembly process. This is supported by the marked increase in yield when higher molar ratios of α to β are used, again paralleling the case of insulin assembly (Frank & Chance, 1983). The failure to achieve renaturation if β-peptide is placed in renaturing conditions in the absence of α-peptide confirms that folded α-peptide is an essential intermediate in the assembly of PA and that assembly depends, like folding, on competition between productive and nonproductive association (Pain, 1978).

The assembly of PA from its constituent unfolded peptides confirms that one cannot generalize from earlier attempts to refold proteins that arise from proteolytic activation. Although cathepsin D (Lah et al., 1984), like pepsin (Ahmed & McPhie, 1978), cannot be renatured after removal of the N-terminal peptide from the zymogen, it has been shown that in the latter enzyme (Privalov et al., 1981), and probably in cathepsin D (Pain et al., 1985), the C-terminal domain can refold but not the N-terminal domain. However, removal of four C-terminal residues from RNase (Taniuchi, 1970) and of six or more C-terminal residues from *E. coli* PA precursor, with a total of 846 residues (Sizmann et al., 1990), prevents folding. In these cases, the information required for folding appears to be lost.

The loss of activity and aggregation of chymotrypsin and insulin on incubation with protein disulfide isomerase (Givol et al., 1965) also shows a loss of the ability of noncovalent interactions to drive the peptide chains spontaneously into the native conformation once activation has taken place. In the case of insulin, however, the A and B chains have been assembled at yields up to 60% [reviewed in Frank and Chance (1983)], showing that the information for correct assembly is present in the activated chains, presumably due to transient formation of unstable folding units or domains. More recently, substantially higher yields have been achieved by using cross-linked insulin. The authors have interpreted this as indicating that the role of the C-peptide in proinsulin is to keep the A and B chains close together during folding (Tang & Tsou, 1990).

Scheme I: Folding and Assembly of PA in Vitro



The results for the folding and assembly of PA can be summarized by Scheme I. α_u and β_u represent the peptides unfolded in denaturant. They can fold to α' and β', respectively, that are able to associate to $\alpha'\beta'$, a state that converts only slowly to native PA $[(\alpha\beta)_N]$. The two peptides are large enough to constitute folding domains in their own right, and this would account for the ability of the α-peptide to fold by itself to a stable conformation (Lindsay & Pain, 1990). The structure of PA precursor can be envisaged as resulting similarly from the association of folding domains.

Alternatively, $\alpha'\beta'$ can aggregate. β' has been shown by urea gradient gel electrophoresis to self-aggregate spontaneously. While at high dilution, β' undergoes a change—here assumed to be a soluble aggregate β_m'' that cannot react with α' to give active PA—it nevertheless will react with α' to form an insoluble aggregate, as shown by the absence of α or β in the supernatant.

The slow kinetics of recovery of activity from a zero value at zero time (Figure 6) show that there is no significant fast formation of active enzyme. This, coupled with the facts that in the absence of association of β with α the former aggregates rapidly and that cross-linking experiments with glutaraldehyde (C. D. Lindsay and R. H. Pain, unpublished work) show the formation of monomeric $(\alpha\beta)$ protein at very early stages in folding, leads to the conclusion that the rate-limiting step in the in vitro assembly process is one of conformational change following association.

Temperature Dependence of Assembly. The sharp fall-off in yield at temperatures above 20 °C is remarkable and represents a temperature-dependent folding and assembly process akin to the folding and assembly of certain mutant trimeric tail spike proteins from *Salmonella typhimurium* (King, 1986). Attention has been drawn to the thermoregulation of PA production in *E. coli* and *Kluyvera citrophila*, by which generation of PA activity is reduced rapidly to zero as the growth temperature is raised from 30 to 37 °C (Valle et al. 1991). A. Böck and C. Keilmann (unpublished work) have carried out in vivo studies of the synthesis, processing, and secretion of PA and have shown that the PA precursor continues to be produced but ceases to be processed or translocated as the temperature is raised above 30 °C.

The in vitro experiments in this paper show that the temperature dependence of folding and assembly is associated with an increased tendency of the α-peptide to aggregate either during or after folding at elevated temperatures. This could be driven by the increasing strength of the hydrophobic in-

teraction as the temperature is raised. This destabilization of the α-peptide in solution accounts for the decrease in productive interaction between the α- and β-peptides in vitro (Figure 2).

If, as the results in this paper suggest, the N-terminal α-peptide portion of the PA precursor folds in the early stages of biosynthesis in vivo of the whole chain allowing the β-peptide portion to fold and rapidly associate with the α-peptide, the temperature dependence of PA processing observed in vivo could result from the temperature-induced instability of folding of the α-peptide moiety. This constitutes a further example of a temperature-dependent block in protein folding in vivo [cf. King (1986)].

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Solution Structure of Fe(II) Cytochrome *c*551 from *Pseudomonas aeruginosa* As Determined by Two-Dimensional ^{1}H NMR†

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ABSTRACT: The solution structure of Fe(II) cytochrome *c*551 from *Pseudomonas aeruginosa* based on 2D ^{1}H NMR data is reported. Two sets of structure calculations were completed with a combination of simulated annealing and distance geometry calculations: one set of 20 structures included the heme–peptide covalent linkages, and one set of 10 structures excluded them. The main-chain atoms were well constrained within the two structural ensembles (1.30 and 1.35 Å average RMSD, respectively) except for two regions spanning residues 30–40 and 60–70. The results were essentially the same when global fold comparisons were made between the ensembles with an average RMSD of 1.33 Å. In total, 556 contraints were used, including 479 NOEs, 53 volume constraints, and 24 other distances. This report represents the first solution structure determination of a heme protein by 2D ^{1}H NMR and should provide a basis for the application of these techniques to other proteins containing large prosthetic groups or cofactors.

Cytochromes are heme (iron porphyrin) proteins generally involved in some type of electron transfer process. Soluble cytochromes are single polypeptides containing one heme group with the iron alternating between Fe(II) and Fe(III) in the electron transfer process. *c*-type cytochromes are distinct in two respects: the heme is covalently attached by two thioether linkages arising from cysteine residues near the N-terminus, and the axial iron ligands are sulfur from methionine and nitrogen from histidine. The spectroscopic properties of *c*-type cytochromes, and heme proteins in general, are well understood (Ochiai, 1977).

Cytochrome *c*551 from *Pseudomonas aeruginosa* is a small bacterial *c*-type cytochrome of 82 residues. We have previously reported the spin system identification, sequential assignments,

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