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Chemical Synthesis, Purification, and Characterization of Two Inflammatory Proteins, Neutrophil Activating Peptide 1 (Interleukin-8) and Neutrophil Activating Peptide 2†

Ian Clark-Lewis,*,‡ Bernhard Moser,§ Alfred Walz,§ Marco Baggiolini,§ George J. Scott,∥ and Ruedi Aebersold‡

The Biomedical Research Centre and Department of Biochemistry, University of British Columbia, Vancouver, British Columbia, Canada V6T 1W5, The Theodor-Kocher Institute, University of Bern, P.O. Box 99, Bern, Switzerland, and The SCIEX Corporation, Thornhill, Ontario, Canada L3T 1P2

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ABSTRACT: Two recently identified pro-inflammatory proteins, namely, neutrophil activating peptide 1 (NAP-1) [also termed interleukin-8 (IL-8)] and NAP-2, were chemically synthesized, purified, and characterized. The fully protected NAP-1/IL-8 (72 residues) and NAP-2 (70 residues) peptide chains were assembled by automated solid-phase methods with average stepwise yields of 99.5 and 99.3%, resulting in overall chain assembly yields of 70 and 62%, respectively. Deprotection resulted in crude products, which were allowed to fold by air oxidation, and were purified by two cycles of reverse-phase high-pressure liquid chromatography, yielding 27 mg of NAP-1/IL-8 and 22 mg of NAP-2. Purity was established by reverse-phase high-pressure liquid chromatography and isoelectric focusing, and the primary structures of the purified products were verified by using mass spectrometry and Edman sequencing methods. Synthetic and recombinant NAP-1/IL-8 were equally active on human neutrophil granulocytes as determined by measuring the induction of cytosolic free calcium, elastase release, and chemotaxis. Synthetic NAP-2 was equivalent to purified natural NAP-2 in the elastase release and calcium mobilization assays, but it was consistently less potent (3–5-fold) as a stimulus of chemotaxis, perhaps indicative of additional chemotactic components in the natural preparation. The results indicate that by chemical synthesis these cytokines can be obtained in purity and quantities suitable for further structural analysis, as well as functional studies both in vivo and in vitro. The ability to rapidly generate analogues with unambiguous primary structure suggests that this will be the method of choice for an in-depth study of structure–function relationships within this family of inflammatory cytokines.

The recruitment and activation of neutrophil leukocytes are central events in the acute inflammatory response. Recently, several members of a family of endogenous human proteins that are potent mediators of these events have been identified (Baggiolini et al., 1989; Matsushima & Oppenheim, 1989). The first protein with these activities to be identified was variously termed neutrophil activating factor (Walz et al., 1987), monocyte-derived neutrophil activating peptide (Schroder et al., 1987), and monocyte-derived neutrophil chemotactic factor (Yoshimura et al., 1987a,b). More recently, the terms neutrophil activating peptide 1 (NAP-1)[1] and interleukin-8 (IL-8) were proposed (Westwick et al., 1989; Leonard, 1990). Other proteins with sequence similarity that suggests structural similarity, including neutrophil activating peptide 2 (NAP-2) (Walz & Baggiolini, 1989) and melanoma

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* To whom correspondence should be addressed.
‡ University of British Columbia.
§ University of Bern.
∥ The SCIEX Corp.

[1] Abbreviations: NAP, neutrophil activating peptide; IL-8, interleukin-8; PBP, platelet basic protein; HPLC, high-pressure liquid chromatography; *t*-Boc, *tert*-butyloxycarbonyl.

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growth stimulatory activity (Richmond et al., 1988), also have neutrophil activating properties (Walz & Baggiolini, 1989; Moser et al., 1990). Other similar proteins, such as platelet factor 4 (Holt et al., 1986), apparently do not influence neutrophil function (Walz et al., 1989).

Originally, NAP-1/IL-8 was isolated from stimulated human mononuclear phagocytes, but subsequently was found to be produced by a wide variety of cell types (Baggiolini et al., 1989). The in vitro effects of NAP-1/IL-8 on neutrophils are similar to those of classical chemotactic agonists, such as C5a or fMet-Leu-Phe, and include induction of a transient rise in cytosolic free calcium concentration, the release of granule enzymes, the respiratory burst, shape change, and chemotaxis (Peveri et al., 1988; Van Damme et al., 1988; Yoshimura et al., 1987a,b; Schroder et al., 1987).

NAP-2 corresponds to the C-terminal 70 amino acids of platelet basic protein (PBP) and shows a similar range of activities to NAP-1/IL-8 (Walz & Baggiolini, 1989). PBP and connective tissue activating peptide III, which is another fragment of PBP, apparently lack neutrophil activating properties (Walz et al., 1990). The production of NAP-2 appears to be mediated by monocyte proteases, following the release of its precursors from the α granules of platelets (Walz & Baggiolini, 1990), a process that renders it quite distinct from NAP-1/IL-8 in terms of cells of origin, generation, and probable site of action.

Thus far, these proteins have been obtained for functional studies by purification from cellular sources, which yields limited quantities, or through recombinant DNA methods (Lindley et al., 1988). These approaches are time-consuming, and difficulties in the expression of its cloned genes are frequently encountered. For example, we have experienced problems in the expression of some NAP homologues (unpublished data). Furthermore, the copurification of contaminants of bacterial origin (e.g., lipopolysaccharides, endotoxins, and cell wall peptides) with the recombinant material is difficult to exclude. This is especially important in studies of this family of cytokines as some bacterial products have overlapping or inducing activities (Schiffmann et al., 1975; Yoshimura et al., 1987a,b; Schroder & Christophers, 1989). Likewise, with biological sources, the potential presence of several biologically and structurally related mediators, and their cleavage products could present problems in their purification.

We have explored the approach of total chemical synthesis to overcome these problems and to begin to address questions of the relationship between structure and function within this family of cytokines. Recent studies have shown that automated chemical synthesis has the potential to provide analogues of proteins in amounts and purity required for structural studies while simultaneously eliminating the possibility of contamination with other biological mediators (Clark-Lewis et al., 1986, 1988a,b, 1989; Woo et al., 1989; Schneider & Kent, 1988; Wlodawer et al., 1989). In addition, this approach has the flexibility of allowing noncoded amino acids and has the speed and reliability to make it efficient and practical.

This paper describes the synthesis, purification, and characterization of NAP-1/IL-8 and NAP-2. Functional comparison of the synthetic cytokines with their recombinant or natural counterparts and structural analysis of the synthetic molecules indicate that this approach is valid and that it will be useful in achieving the goals of structure–function analysis and the eventual engineering and redesign of these molecules.

Materials and Methods

Chemical Synthesis Materials. N^{α}-*tert*-Butyloxycarbonyl (*t*-Boc)-amino acids were obtained from the Peptide Institute, Osaka, Japan, except N^{α}-*t*-Boc-N^{im}-(dinitrophenyl)histidine which was obtained from Bachem Inc., Switzerland. Trifluoroacetic acid was purchased glass-distilled from Halocarbon Inc., North Augusta, SC, and redistilled before use. Dimethylformamide was from Burdick and Jackson, Muskegon, MI, after selected lot numbers were pretested. Dichloromethane was from BDH Canada, Toronto, Ontario, after selected lot numbers were pretested. Methanol was glass-distilled-grade from BDH Canada. *N*,*N*-Diisopropylethylamine, *N*,*N*-dicyclohexylcarbodiimide, 1-hydroxybenzotriazole, and the pam resins were from Applied Biosystems, Foster City, CA. Hydrogen fluoride was from Matheson Canada, Edmonton, Canada, and *p*-cresol, *p*-thiocresol, and dimethyl sulfide were from Aldrich Chemical Co., Milwaukee, WI.

Chemical Synthesis Methods. NAP-1/IL-8 and NAP-2 were synthesized by using solid-phase methods (Merrifield, 1963) that were optimized and adapted to a fully automated peptide synthesizer (Applied Systems 430A) and described in detail elsewhere (Clark-Lewis et al., 1986; Clark-Lewis & Kent, 1989; Kent, 1988). The synthesis was started with the protected C-terminal amino acid linked to a cross-linked polystyrene resin via a 4-(carboxamidomethyl)benzyl ester linkage (the so-called pam resin) (0.4 mmol of 0.8 mmol/g of aminoacyl resin). N^{α}-*t*-Boc acids with appropriate side chain protecting groups were added in a stepwise fashion until the entire protected polypeptide chain was formed. Side chain protection was as follows: benzyl (Asp, Glu, Ser, and Thr); 4-methylbenzyl (Cys); toluenesulfenyl (Arg); 2-chlorobenzyloxycarbonyl (Lys); 2-bromobenzyloxycarbonyl (Tyr); formyl (Trp); dinitrophenyl (His); and none (Ala, Asn, Gly, Gln, Ile, Leu, Met, Phe, Pro, Val). Samples were automatically taken after each step to retrospectively monitor the amino acid coupling yields using a ninhydrin-based reaction (Sarin et al., 1981).

The protected polypeptide resin was treated twice for 30 min with 2-mercaptoethanol (20%) in dimethylformamide containing diisopropylethylamine (5%) to remove the DNP groups from the histidine side chains. The resin was dried and cleaved by using the "low–high" hydrogen fluoride method as described (Tam et al., 1983) except for the following modifications. After the 25% hydrogen fluoride step, the partially protected peptide resin was filtered from the reaction mixture by using an all-Teflon filtration apparatus fitted with a Zitex filter and washed with dichloromethane and dried before the high 90% hydrogen fluoride step. The ethyl acetate precipitate of the material released from the resin was dissolved in 50 mL of 6M guanidine hydrochloride, 0.1 M Tris–acetate, pH 8.5, and 20% 2-mercaptoethanol and stirred at 37 °C for 2 h and then acidified with 2 mL of acetic acid. This mixture was termed the crude peptide product.

The cost of generating the crude product using the procedure outlined was approximately $1100.00 ($15.00 per amino acid plus resin). The time taken was 6 days. From our experience, generation of material by conventional recombinant DNA technology would have been at least as expensive and would have taken several months. However, it should be noted that the synthesis procedure is not easily scaled up, so for generation of large (greater than 25 mg of pure product) quantities of material, DNA expression may be more economical.

HPLC Purification and Folding. Three different C-18 silica columns were used in the purification and analysis of the synthetic NAP-1/IL-8 and NAP-2 proteins. They were a preparative column (22.4 × 250 mm column with a 22.4 × 100 mm guard column) packed with 12-μm, 300-Å pore size

packing (Dynamax, Rainin Instrument Co., Woburn, MA.); a semipreparative (10 × 250 mm) Vydac C-18 column, with 5-μm particle, 300-Å pore-size packing (Separations Group, Hesperia, CA), and an analytical 4.6 × 250 mm column (Vydac) containing the same packing. The crude peptide product was loaded onto the preparative column and the retained material eluted with a 0–60% water–acetonitrile gradient in 0.1% trifluoracetic acid over 4 h at a flow rate of 15 mL/min. A sample (25 μL) of fractions containing 225-nm UV-absorbing material was rerun on the analytical column, and by comparison with the profile of the crude material, fractions containing the major peak were pooled and lyophilized. This material was reconstituted in 1 M guanidine hydrochloride and Tris–acetate, pH 8.5, at a concentration of 0.2 mg/mL and stirred vigorously overnight in an open beaker so that air was kept bubbling through the mixture by vortex action. This procedure has been found to promote formation of the disulfide bridges by oxidation of the appropriate half-cysteines (Clark-Lewis et al., 1988a; Woo et al., 1989). This material was acidified with 2 mL of acetic acid, and half was loaded onto the semipreparative column and the retained material eluted with the same gradient as before at a flow rate of 3 mL/min. Samples of each fraction were run on the analytical column. Fractions containing only material with the retention time of the major peak in the folded material were pooled and lyophilized as purified NAP-1/IL-8 or NAP-2.

Isoelectric Focusing. Mini polyacrylamide gels (Pharmacia "PHAST" gels, IEF 3-9; Pharmacia, Uppsala, Sweden) were washed in 8 M urea and then in 8 M urea containing pH 9–11 Ampholytes (Pharmacia), for 30 min each, either with or without 10 μg/mL dithiothreitol. Gels were prerun for 15 V·h at 200-V, 2.0-mA, 3.0-mW maximum settings, and the samples were loaded and run for 410 V·h at 1000-V, 5.0-mA, 3.0-mW maximum settings on the Pharmacia "PHAST" system for a total of 500 V with maximum settings of 2.0 mW, 5.0 mA, and 1000 V. The pH gradient was determined by using a surface pH electrode. The gels were stained with silver by using the "PHAST" developing system as described in the manual.

Mass Spectrometry. Ion spray mass spectrometry was carried out on a SCIEX triple quadrupole mass spectrometer equipped with a liquid delivery apparatus. The molecular mass from the peaks corresponding to the different charge to mass ratios of the different multiple ionized species of the protein was analyzed as described (Covey et al., 1988).

Protein Sequencing. Protein sequences were determined by Edman degradation using either solid-phase or gas–liquid-phase methods. For solid-phase sequence analysis, reduced and carboxymethylated protein or proteolytic cleavage fragments were coupled to arylamine-functionalized poly(vinylidene difluoride) membranes. (Sequelon AA; Milligen/Biosearch, Burlington, MA) using the water-soluble carbodiimide 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride (Aebersold et al., 1990; Coull et al., 1991) and sequenced in a Milligen/Biosearch Model 6600 sequencer using standard protocols (Coull et al., 1991).

For gas–liquid-phase sequence analysis, polypeptides were applied to Polybrene-coated glass fiber disks and sequenced in an Applied Biosystems Model 477 protein sequencer using standard protocols. Sequencing of protected peptide resins was carried out on N^{α}-deprotected samples by using the same methods.

N-Terminal solid-phase sequencing runs revealed a major portion of the sequence. The remaining sequence was obtained by runs of HPLC-fractionated fragments, derived either by proteolytic cleavage with Asp-*N*-endoprotease (Boehringer Mannheim Canada, Laval, Quebec) or by chemical cleavage, through preferential hydrolysis of the Asp–Pro peptide bond in dilute formic acid.

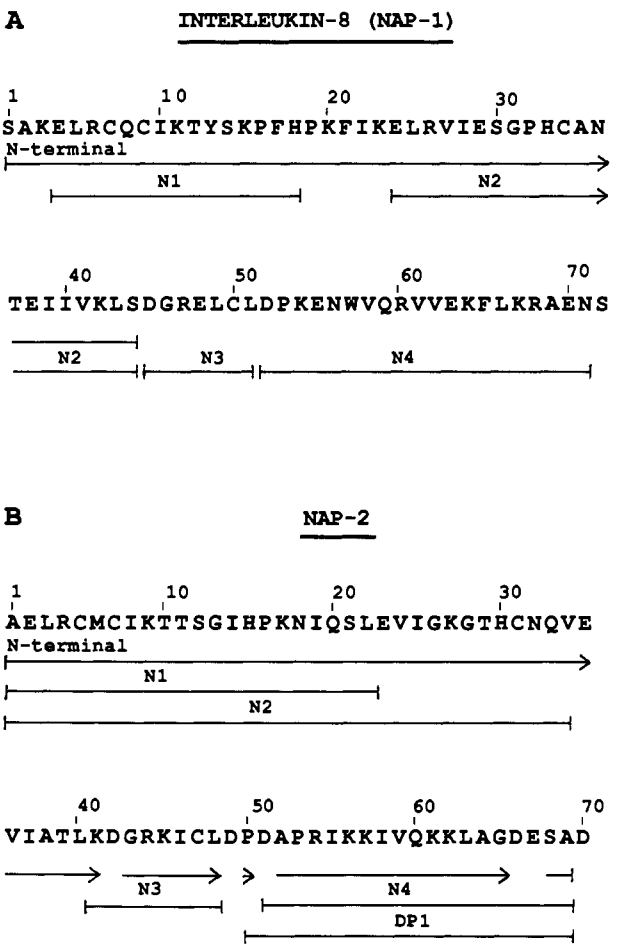

FIGURE 1: Sequence and sequence analysis of purified synthetic NAP-1/IL-8 and NAP-2. NAP-1/IL-8 (panel A) and NAP-2 (panel B) were synthesized according to the sequences described; NAP-1/IL-8 (Lindley et al., 1988; Matsushima et al., 1988) and NAP-2 (Walz & Baggiolini, 1989). The synthetic proteins were reduced and carboxymethylated and sequenced by Edman degradation methods. In panels A and B, the line labeled "N-terminal" represents sequence derived from N-terminal sequence analysis; and N1–4 represents sequence derived from Asp-*N*-endoprotease cleavage fragments. In panel B, DP1 represents the sequence of the cleavage fragment resulting from preferential acidolysis of the Asp–Pro peptide bond.

Biological Assays. Human neutrophils were isolated from buffy coats of donor blood as described (Peveri et al., 1988). The final suspension, 10^8 cells/mL, was kept in 0.15 mM NaCl–0.05 mM $CaCl_2$ at 10 °C until use. Elastase release, cytosolic free calcium concentration changes, and chemotaxis were determined according to published methods (Peveri et al., 1988; Schroder et al., 1987).

Recombinant and Natural Proteins. Recombinant NAP-1/IL-8 was obtained from the Sandoz Research Institute, Vienna, Austria. NAP-2 was purified from human buffy coat leukocytes as described previously (Walz & Baggiolini, 1989).

RESULTS

Synthesis of IL-8/NAP-1 and NAP-2. Fully protected IL-8/NAP-1 (72 residues) and NAP-2 were assembled on cross-linked polystyrene resins according to the amino acid sequences shown in Figure 1. In each case, the chain assembly process was continuous and took 5 days to complete. Samples were automatically taken at each step to document, postsynthetically, the extent of the coupling reactions throughout the

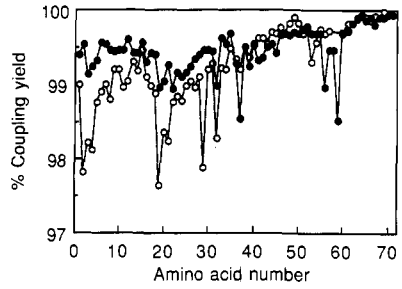

FIGURE 2: Amino acid coupling yields for NAP-1/IL-8 and NAP-2. Samples for residues 58 and 59 of NAP-2 were not taken. Yields are calculated from the level (millimoles per gram) of residual unreacted primary amine after coupling which is determined postsynthetically by analysis of peptide resin samples using ninhydrin.

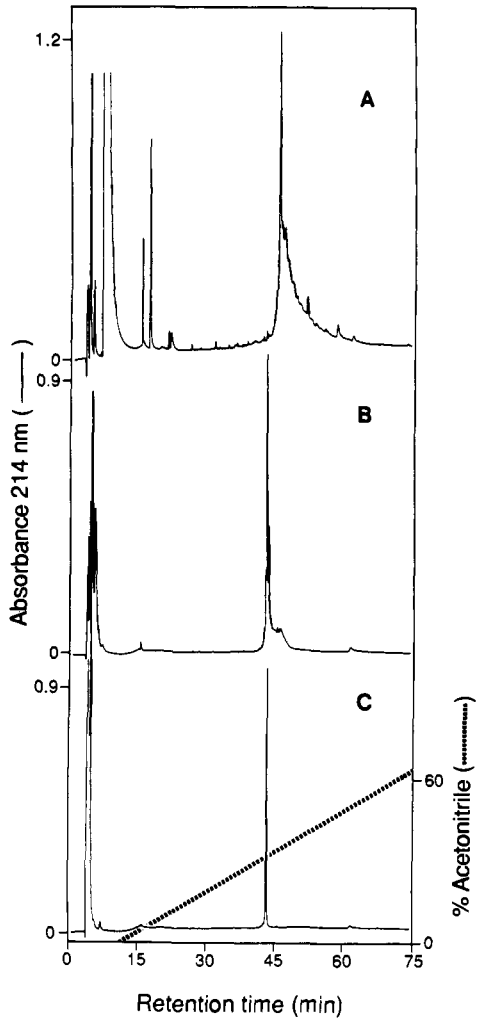

FIGURE 3: Analytical reverse-phase HPLC chromatograms of NAP-1/IL-8 at various stages of purification. Shown is the crude peptide product (panel A), folded material after preparative HPLC (panel B), and the final purified NAP-1/IL-8 after semipreparative HPLC (panel C).

synthesis. As shown in Figure 2, the stepwise yields were in the 97–100% range with an average of 99.5% per step for NAP-1/IL-8 and 99.3% per step for NAP-2. The data were not corrected for the residual background, which is known to steadily increase with increasing chain length, because it is difficult to accurately quantify (Clark-Lewis & Kent, 1989). Nevertheless, difficult sequences, as defined by a series of coupling yields below 95% (Meister & Kent, 1984), were not encountered in either synthesis.

In the case of NAP-1/IL-8, the synthetic yields were confirmed by Edman degradation sequencing of the protected

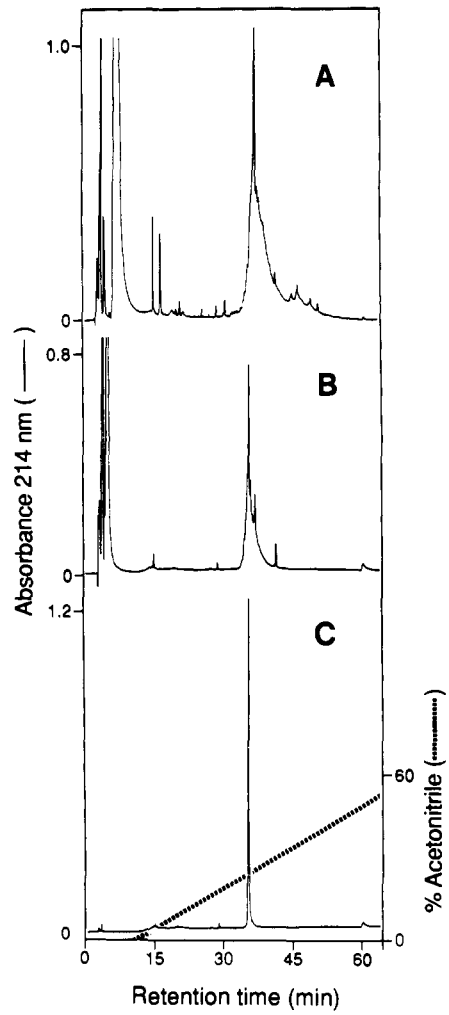

FIGURE 4: Analytical reverse-phase HPLC chromatograms of NAP-2 at various stages of purification. Shown is the crude peptide product (panel A), folded material after preparative HPLC (panel B), and the final purified NAP-2 after semipreparative HPLC (panel C).

peptide resin and an intermediate sample of 30–72 peptide resin. The rationale for determining yields by measuring the cumulative premature appearance "preview" during Edman degradation sequencing has been discussed in detail elsewhere (Kent et al., 1982; Clark-Lewis & Kent, 1989). The values of preview from residues 1–32 and 30–66 were 13.3 and 19.2%, respectively. Taking into account overlaps, this was calculated to give an average stepwise yield of 99.5% for residues 1–66. The results of the ninhydrin assays and the Edman sequencing method for NAP-1/IL-8 were comparable. As this has been a consistent finding in past syntheses [see Clark-Lewis and Kent (1989)], we did not consider it necessary to carry out the preview determination for NAP-2.

Taken together, these results indicate that for NAP-1/IL-8 and NAP-2 the yields of correctly assembled chains were estimated to be 70% and 62%, respectively.

Purification of the Synthetic Proteins. A summary of the syntheses and purification yields is shown in Table I. After the protected polypeptide chains had been assembled, they were deprotected and released from the resin by using hydrogen fluoride (see Materials and Methods). This resulted in a crude product containing the desired polypeptide and various byproducts of the synthesis the nature of which has been discussed elsewhere (Tam et al., 1983; Kent, 1988; Clark-Lewis & Kent, 1989). Analysis by reverse-phase HPLC on a C-18 column revealed a major peak and byproducts that elute predominantly after the main peak (NAP/IL-8, Figure 3A; NAP-2, Figure 4A). The denatured and reduced material

Table I: Summary of Yields[a]

	NAP-1/IL-8 (mg)	NAP-2 (mg)
resin (0.4 mmol)	563	571
full-length protected peptide resin[b]	3280	3030
crude product	1500	1400
preparative HPLC folding	190	150
semipreparative HPLC[c]	27	22

[a] Yields are calculated for the entire synthesis. [b] The full 0.4 mmol is not recovered because samples of peptide resin are taken during the synthesis. [c] Estimated by weighing a sample following dialysis and lyophilization.

was loaded onto a preparative C-18 reverse-phase HPLC column. The eluted fractions were individually analyzed to assess their content of the major peak identified in the crude material (Figures 3A and 4A). Those apparently containing 80% or more of the major peak were pooled and lyophilized. Approximately 170 mg was obtained at this stage (Table I). The disulfide bridges were formed by gentle air oxidation (see Materials and Methods), and analytical HPLC of the folded partially purified products is shown for NAP-1/IL-8 in Figure 3B and NAP-2 in Figure 4B. In both cases, the folded material eluted more than 1 min earlier than the fully reduced material. A similar pattern was observed for correctly folded synthetic human transforming growth factor α (Woo et al., 1989). Fifty percent of the folded material was loaded onto a semipreparative reverse-phase C-18 column, and samples of the resulting fractions were analyzed for the presence of the major peak of folded product. Fractions containing only the folded products were pooled and lyophilized (yields are shown in Table I) and were designated NAP-1/IL-8 and NAP-2.

Purity of the Synthetic Proteins. Analytical HPLC profiles of the purified NAP-1/IL-8 and NAP-2 are shown in Figures 3C and 4C, respectively. In each case, a single symmetrical peak was observed, thus demonstrating that the two preparative HPLC steps were effective in isolating the major component as detected by analytical reverse-phase HPLC. To more thoroughly assess the purity of the HPLC-purified material, an analytical technique distinct from that used in the purification was required. Isoelectric focusing was chosen based on past experience which indicated that this method had the resolving power to separate closely related contaminants that could be present in synthetic preparations (Clark-Lewis & Kent, 1989; Woo et al., 1989). The results shown in Figure 5 indicate that under nonreducing conditions both NAP-1/IL-8 and NAP-2 behave as single components with isoelectric points of 9.4 and 9.6, respectively. The focusing was somewhat diffuse, and the proteins were difficult to stain under nonreducing conditions. Under reducing conditions in the presence of dithiothreitol, the bands were sharper and the staining more intense, but, again, only a single component was detected even at higher loadings than those shown in Figure 5.

Structural Analysis. The purified materials were further characterized to establish the authenticity and purity of the synthetic product. To provide estimates of the molecular masses of the synthetic proteins, high-mass ion-spray mass spectrometry was used. As described elsewhere, this technique leads to multiple ionized species and allows mass estimation of high resolution and accuracy (Covey et al., 1988). The mass spectra of NAP-1/IL-8 and NAP-2 are shown in Figure 6A,B. When analyzed, these data yielded mass estimates of 8381.6 and 7624.1 for NAP-1/IL-8 and NAP-2, respectively. Within the 0.1% accuracy range expected of this technique, these estimates were consistent with the expected molecular masses of the folded proteins which were calculated to be 8385.7 and

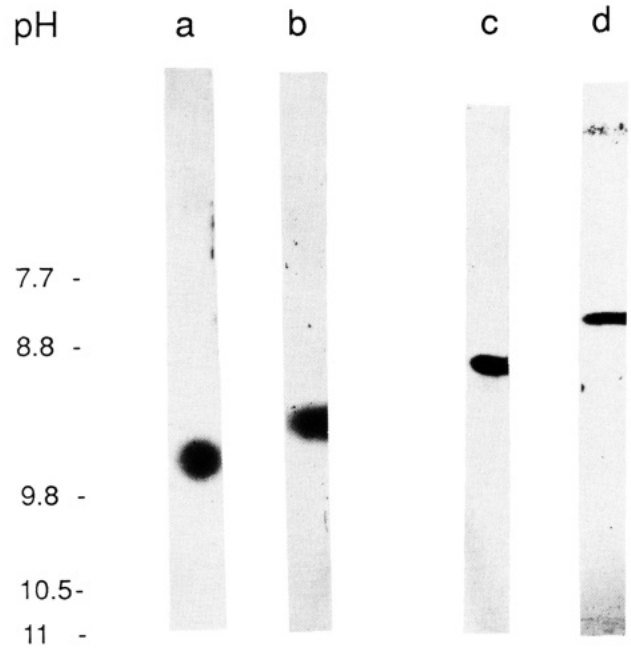

FIGURE 5: Isoelectric focusing of purified NAP-1/IL-8 and NAP-2. Silver-stained analytical isoelectric focusing gels of NAP-1/IL-8 nonreduced (lane a) and reduced (lane b) and of NAP-2 nonreduced (lane c) and reduced (lane d).

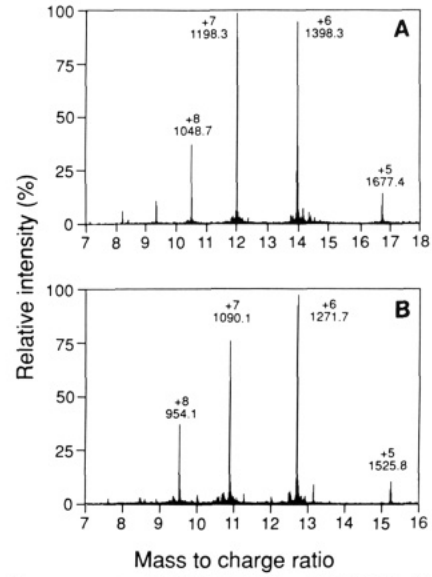

FIGURE 6: Mass spectra of NAP-1/IL-8 and NAP-2. NAP-1/IL-8 (panel A) and NAP-2 (panel B) were loaded and ionized by using ion spray techniques. Indicated are the charges and the charge to mass ratio of each of the multiple ionized species.

7626.9 for NAP-1/IL-8 and NAP-2, respectively.

Sequence Analysis. The purified proteins were analyzed for amino acid composition, and the results were consistent with those expected for molecules with the sequence described in Figure 1 (data not shown). To more definitely establish the structure of the synthesized molecules, they were sequenced by using stepwise Edman chemistry. N-Terminal sequencing of the intact proteins provided more than half the sequence information of synthetic NAP-1/IL-8 and essentially the total sequence of NAP-2. The remainder was determined and confirmed by sequencing peptide fragments isolated after enzymatic or chemical cleavage of the proteins (Figure 1A,B). The complete sequences of the purified synthetic proteins verified their primary structures to be those described for NAP-1/IL-8 (Lindley et al., 1988; Matsushima et al., 1988) and NAP-2 (Walz & Baggiolini, 1989).

Biological Analysis. The ability of the synthetic NAP-

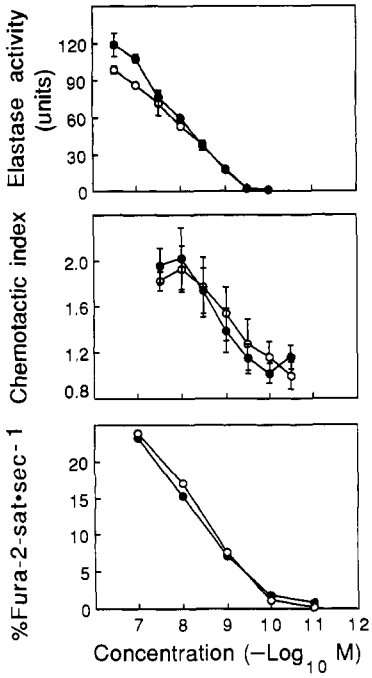

FIGURE 7: Activites on purified neutrophils of the synthetic NAP-1/IL-8. Synthetic (●) and *E. coli* derived (○) NAP-1/IL-8 was assayed for induction of elastase release ± SD (top panel), neutrophil chemotaxis ± SD (middle panel), and cytosolic free calcium concentration (bottom panel).

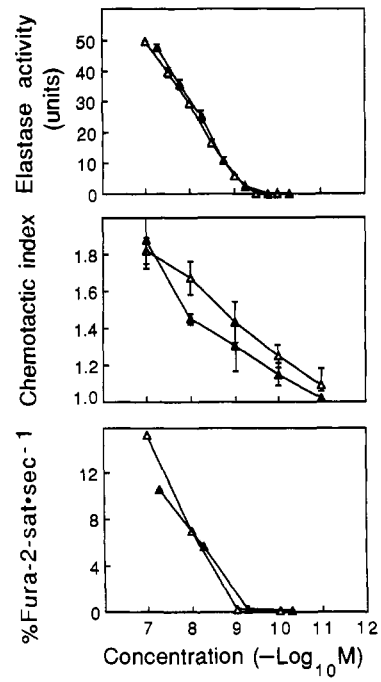

FIGURE 8: Activites on purified neutrophils of the synthetic NAP-2. Synthetic (▲) and purified natural (△) NAP-2 was assayed for induction of elastase release ± SD (top panel), neutrophil chemotaxis ± SD (middle panel), and cytosolic free calcium concentration (bottom pannel).

1/IL-8 and NAP-2 preparations to activate human neutrophil granulocytes was tested in assays for release of elastase (exocytosis), chemotaxis, and mobilization of cytosolic free calcium. The relative potencies of the synthetic molecules were compared to the activities of recombinant NAP-1/IL-8 and native NAP-2. A recent comparative analysis of recombinant and native NAP-1/IL-8 demonstrated that their activities and potencies were similar (Lindley et al., 1988). As shown in Figure 7, synthetic NAP-1/IL-8 exhibited virtually the same activity profile as recombinant (and therefore also as native) NAP-1/IL-8. Although structurally homologous, native NAP-2 clearly differs from synthetic/recombinant NAP-1/IL-8 in its potency to stimulate neutrophils (compare Figures 7 and 8), confirming previous results (Walz et al., 1989). The synthetic NAP-2 preparation was close to indistinguishable from native NAP-2 in assays for the release of elastase (Figure 8, top panel) and changes in the internal free calcium concentrations (Figure 8, bottom panel). However, when titrated in assays for chemotaxis, the synthetic NAP-2 had a consistently lower activity (3–5-fold when 50% of the maximal response was compared) than the native preparation (Figure 8, middle panel). The data in Figures 7–9 are representative of two to four independently conducted experiments using neutrophils from the blood of different donors.

In the above experiments, it was evident that NAP-1/IL-8 was more active than NAP-2, in terms of both the maximal response and the concentration required for 50% of the maximal response. However, the responsiveness of different neutrophil preparations varies widely, so it is not possible to reliably compare different assays. In the experiment shown in Figure 9, the synthetic NAP-1/IL-8 was compared with synthetic NAP-2 in the same assay, and the data show that NAP-1/IL-8 was significantly more potent on a molar basis than NAP-2, particularly in the elastase release assay.

Discussion

In this study, two cytokines, NAP-1/IL-8 (72 residues) and NAP-2 (70 residues), were chemically synthesized by using

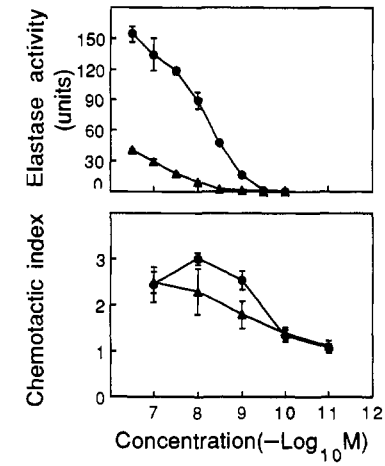

FIGURE 9: Comparison of synthetic NAP-1/IL-8 and NAP-2. Shown is activity of synthetic NAP-1/IL-8 (●) and NAP-2 (▲) on purified human neutrophils in assays for induction of elastase release ± SD (top panel) and chemotaxis ± SD (bottom panel).

automated solid-phase methods. The proteins were folded, purified to apparent homogeneity by reverse-phase HPLC, and sequenced to verify their primary structure. Both cytokines were fully active in assays which measured neutrophil elastase release and in the mobilization of intracellular free calcium. Taken together, the results indicate that the synthetic proteins are pure and authentic.

The synthetic NAP-1/IL-8 had activity indistinguishable from recombinant NAP-1/IL-8 expressed in *Escherichia coli* (Lindley et al., 1988). In previous studies, it was demonstrated that the recombinant NAP-1/IL-8 was equivalent in activity to that purified from natural sources (Lindley et al., 1988). Thus, all three preparations were equivalent in the assays used so far. The data suggest that it will be possible to reliably establish structure–function correlations by comparison of defined synthetic analogues. This confidence in part stems from the detailed analysis we have performed in this study.

In this regard, another report (Tanaka et al., 1988) of the synthesis of IL-8 was difficult to evaluate because of lack of characterization of the product to establish purity and structure.

The chemical synthesis approach described here circumvents the potential problems arising from undesirable posttranslational and postsecretory processing of recombinant proteins by host organisms. For example, in contrast to NAP-1/IL-8, the gene for NAP-2 has not yet been expressed in *E. coli*. We have experienced difficulties in obtaining stable *E. coli* clones that produce recombinant NAP-2 (unpublished observations). Therefore, at present, chemical synthesis is the only means of generating purified NAP-2, other than the limited amounts that can be isolated from blood cells. Clearly these expression problems could become even more critical when analogues with differing primary sequences are required.

The synthetic NAP-2 was equal to natural NAP-2 as an inducer of elastase release and cystolic free calcium, but was less active as a stimulus stimulating neutrophil chemotaxis. The reason for the difference cannot be determined with certainty but could be due to reduced activity of the synthetic material or alternatively elevated activity of the native preparation. However, it is unlikely to be due to the presence, in the synthetic form, of the incomplete or aberrant formation of disulfide bridges, or other contaminants from the synthesis, as such byproducts are well separated and easily purify away from the major form which is fully active in the other assays. Considering the high purity of the synthetic protein preparation, this discrepancy may be attributable to chemotactically active impurities in the natural NAP-2 preparation.

The availability of synthetic NAP-2 will allow further studies of this newly discovered cytokine. However, the chemical synthesis approach could also be used to identify and ascribe functional activity to other members of this protein family. For example, in a separate study, the synthesis of melanocyte growth stimulating activity, which also shares structural similarity to the neutrophil activating protein family, enabled the identification of its neutrophil activating properties both in vivo and in vitro (Moser et al., 1990). In vivo studies of inflammatory cytokines require milligram quantities of material free of pyrogens and contaminating proteins, a requirement that is fulfilled by the synthetic approach. Chemical synthesis may also shed light on the possible functional heterogeneity of the different N-terminally processed forms of NAP-1/IL-8. Furthermore, chemical synthesis may allow testing of predictions regarding the possible existence of new members of this family of mediators which are yet to be described. For example, the following question could be asked: Can platelet factor 4 be processed to give rise to active fragments in a way analogous to platelet basic protein, or alternatively, what are the biological actions of the putative product of the interferon-induced γIP-10 gene (Luster et al., 1985), identified by cDNA cloning?

NAP-1/IL-8 and NAP-2 were synthesized and purified in a period of 2 weeks to give material in sufficient quantities, and in a form suitable for immediate analysis. This study has implications for approaches to protein structure and function relationships where multiple analogues are required and where the ability to generate large numbers of analogues with unambiguous primary structure is required. In terms of consumable costs, the peptide synthesis approach is not considerably more expensive than the recombinant DNA approach. However, the peptide synthesis approach cannot be easily scaled-up and is likely to be useful for structure–function studies rather than large-scale production. In choosing between the two approaches, a key question is which one can supply the required amount of pure material in the optimal time frame. Clearly, length is a critical factor with the peptide synthesis approach as this will affect the ability to purify the desired product. With the synthesis strategy used in this study, it seems that an average of 100–110 residues is about the maximal length for which a homogeneous product in satisfactory yield can be obtained (Clark-Lewis, unpublished observation). All else being equal, the potential usefulness of noncoded amino acids is another consideration. In contrast to recombinant techniques, automated synthesis allows the flexibility of easily incorporating nonnatural and unusual chemical species into sequences, thus increasing the scope of possible changes. Furthermore, rapidly developing methods such as heteronuclear proton NMR of proteins may well require specific isotopic labeling of amino acids (Oschkinat et al., 1988). In this regard, a three-dimensional structure in solution of NAP-1/IL-8 has recently been described (Clore et al., 1990). Thus, the chemical synthesis of NAP-1/IL-8 and NAP-2 analogues greatly expands the potential for detailed analysis of the structure and function within this important family of inflammatory protein mediators.

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Porcine Pancreatic Phospholipase A_2: Sequence-Specific ^1H and ^{15}N NMR Assignments and Secondary Structure†

Niek Dekker,‡ Anton R. Peters,§ Arend J. Slotboom,‡ Rolf Boelens,§ Robert Kaptein,*,§ and Gerard de Haas‡

Center for Biomembranes and Lipid Enzymology and Bijvoet Center for Biomolecular Research, State University of Utrecht, P.O. Box 80.054, 3584 CH Utrecht, The Netherlands

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ABSTRACT: The solution structure of porcine pancreatic phospholipase A_2 (124 residues, 14 kDa) has been studied by two-dimensional homonuclear ^1H and two- and three-dimensional heteronuclear ^{15}N-^1H nuclear magnetic resonance spectroscopy. Backbone assignments were made for 117 of the 124 amino acids. Short-range nuclear Overhauser effect (NOE) data show three α-helices from residues 1-13, 40-58, and 90-109, an antiparallel β-sheet for residues 74-85, and a small antiparallel β-sheet between residues 25-26 and 115-116. A ^{15}N-^1H heteronuclear multiple-quantum correlation experiment was used to monitor amide proton exchange over a period of 22 h. In total, 61 amide protons showed slow or intermediate exchange, 46 of which are located in the three large helices. Helix 90-109 was found to be considerably more stable than the other helices. For the β-sheets, four hydrogen bonds could be identified. The secondary structure of porcine PLA in solution, as deduced from NMR, is basically the same as the structure of porcine PLA in the crystalline state. Differences were found in the following regions, however. Residues 1-6 in the first α-helix are less structured in solution than in the crystal structure. Whereas in the crystal structure residues 24-29 are involved both in a β-sheet with residues 115-117 and in a hairpin turn, the expected hydrogen bonds between residues 24-117 and 25-29 do not show slow exchange behavior. This and the absence of several expected NOEs implie that this region has a less well defined structure in solution. Finally, the hydrogen bond between residues 78-81, which is part of a β-sheet, does not show slow exchange behavior.

Phospholipase A_2 (PLA[1], EC 3.1.1.4) is a calcium-dependent enzyme that specifically cleaves the 2-acyl linkage of *sn*-3-phosphoglycerides (Waite, 1987). Phospholipases occur both extra- and intracellularly. The extracellular PLA's from mammalian pancreas and from snake venom (Waite, 1987;

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* Address correspondence to this author.
‡ Center for Biomembranes and Lipid Enzymology.
§ Bijvoet Center for Biomolecular Research.

[1] Abbreviations: PLA, phospholipase A_2; NMR, nuclear magnetic resonance; COSY, correlated spectroscopy; NOE, nuclear Overhauser enhancement; NOESY, nuclear Overhauser enhancement spectroscopy; TOCSY, total correlation spectroscopy; HOHAHA, homonuclear Hartmann-Hahn; HMQC, heteronuclear multiple-quantum correlation spectroscopy; 2D, two dimensional; ppm, parts per million; TPPI, time-proportional phase increment; SCUBA, stimulated cross-peaks under bleached α's; TMS, tetramethylsilane.

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