FOR THE RECORD

Chemical modification of a variant of human MIP-1 α ; implications for dimer structure

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Abstract: A sequence variant of human MIP-1 α , in which Asp26 has been replaced by Ala, has been chemically modified by the addition of ¹³C-labeled methyl groups at each of the lysine residues and the N-terminus. The sites of methylation have been verified by a combination of MALDI-TOF mass spectrometric experiments and tryptic digestion followed by N-terminal mapping. The effect of the modification on the structure and activity of the protein have been determined by analytical ultra-centrifugation, ¹³C NMR spectroscopy and receptor binding studies. The results of these experiments suggest that huMIP-1 α D26A (BB10010), when present as a dimer, adopts a globular structure, like MCP-3, rather than the elongated or cylindrical structure determined for dimers of huMIP-1 β and RANTES.

Keywords: ¹³C NMR; chemical modification of lysines; human MIP-1 α

Human MIP-1 α is a member of a large family of proteins called the chemokines (Creighton, 1993). Among this family, molecular weights vary between 7–10 kDa, and sequence homologies vary between 20 and 45%. A common feature of chemokines is the presence of four cysteine residues, forming two disulfide bridges, which play a significant role in stabilizing the protein structure.

The chemokine family may be subdivided into two major and two minor classes, depending on the sequence arrangement of the first two cysteines. In the first (major) class, the MIP-2 or α -chemokines, the cysteines are separated by a single amino acid residue (CXC), in the second (major) class, the MIP-1 or β -group, the cysteines follow each other in sequence (CC). The minor sub-

division of chemokines includes the CX₃C and C variants (Proudfoot, 1998). Human MIP-1 α is a member of the second major subclass.

Since their discovery in the early 1980s, there has been considerable biochemical and pharmaceutical interest in the chemokines. They have been implicated in inflammatory response and cell growth regulation (Ward & Westwick, 1998). The α -chemokines are potent chemoattractants for neutrophils (Wolpe & Cerami, 1989) and T-cells (Loetscher et al., 1996). In contrast, the β -chemokines are chemoattractants for monocytes (Walz et al., 1987; Baggiolini et al., 1994), basophils (Bischoff et al., 1993), and eosinophils (Rot et al., 1992). Among the best characterized of the chemokines are IL-8 (Clöre et al., 1990; Clöre & Gronenborn, 1991), NAP-2 (Malkowski et al., 1995), and PF4 (St. Charles et al., 1989; Zhang et al., 1994b)—all α -chemokines; and human MIP-1 α (LD78) (Czaplewski et al., 1999), human MIP-1 β (ACT2) (Lodi et al., 1994), MCP-3 (Meunier et al., 1997), and RANTES (Chung et al., 1995), human CC-2 (Sticht et al., 1999)—all β -chemokines. A number of these molecules have had their structures determined by X-ray crystallography and/or NMR spectroscopy, and a pattern seems to have emerged from these studies. The structures of the monomeric units of a range of chemokines have been found to be very similar, but differences have emerged in the higher order structures that subsequently form. The importance of higher order structures in terms of the function of these molecules has recently been questioned (Laurence et al., 2000). Nevertheless, there is a trend that suggests that the α - or CXC chemokines, such as IL-8 (Clöre et al., 1990), form globular dimers, whereas the β - or CC chemokines, such as RANTES (Chung et al., 1995) and huMIP-1 β (Lodi et al., 1994), form elongated dimers. However, there are some notable exceptions to this trend. MCP-3, a CC chemokine, has been observed to form a globular structure (Meunier et al., 1997) and PF4, a CXC chemokine, preferentially forms a tetramer (Zhang et al., 1994b). Differences in higher order structures of the chemokines has been attributed to differences in the distribution of surface hydrophobicities (Covell et al., 1994). Different quaternary structures for essentially identical monomers is a rare occurrence;

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Abbreviations: MALDI-TOF, matrix assisted laser desorption ionization–time of flight; MIP, macrophage inflammatory protein.

only a few appear in the literature (Royer et al., 1985; Shaanan et al., 1991).

Recently, the solution structure of monomeric huMIP-1 α and its sequence variant, huMIP-1 α D26A, has been determined using NMR spectroscopy (Czaplewski et al., 1999). These structures, as expected, show striking similarities with those of other chemokines. In common with similar studies of a range of chemokines, large errors were observed when trying to converge on a structure for the dimeric form of the protein. Consequently, there is considerable uncertainty in the structure of the monomer–monomer interface. As part of a series of studies to establish the state of multimerization of huMIP-1 α , and sequence variants thereof (Ashfield, 1997), under a range of experimental conditions, we have chemically modified one of these variants, huMIP-1 α D26A. We had previously found that as with PF4, both huMIP-1 α , and huMIP-1 α D26A undergo pH-induced multimerization (Ashfield, 1997). HuMIP-1 α D26A was found to be stable as a dimer over a wider pH range than the wild-type protein, and this was one reason for carrying out further structural studies on this variant (Ashfield, 1997; Czaplewski et al., 1999). Unlike PF4 (St. Charles et al., 1989; Zhang et al., 1994b), huMIP-1 α D26A was observed to undergo multimerization, which was fast on the NMR proton chemical shift timescale, apparently precluding the use of NMR to follow aggregation events. We determined that the timescale for aggregation, at least at the dimer-to-tetramer stage, could not be significantly changed by changing sample buffers. Therefore, chemical modification of surface residues might help resolve this problem. In addition, chemical modification offered the opportunity to determine not only which sites were important for high-order aggregation but also to introduce an NMR label for structure probing. Lysine modification was reported to be the most feasible and predictable (Lundblad, 1995) methods for protein modification. Lysines may be modified in a variety of ways (Muller, 1981; Nieto & Palacian, 1988; Yamashita et al., 1993). Alkylation, specifically methylation, was opted for as a way of introducing relatively small chemical groups (Jentoft & Dearborn, 1979; Zhang et al., 1994a). A series of experiments were performed to establish which residues had been labeled and the consequences of these for the activity of the protein. An interesting observation arose during the course of the experiments that is consistent with the structure for huMIP-1 α D26A dimer, under the buffer conditions we have used, being globular.

Results and discussion: Human MIP-1 α was one of the first chemokines to be identified (Obaru et al., 1986). It is a 69-residue protein with >70% sequence homology with ACT2 (huMIP-1 β) (Lipes et al., 1988). The ^1H NMR spectra for huMIP-1 α and huMIP-1 α D26A, at low pH, are almost identical, suggestive of similar structures (as recently determined (Czaplewski et al., 1999)). Analytical ultracentrifugation data have previously determined that at low pH values both proteins exist predominantly in dimeric forms (Patel et al., 1993). Mutant and wild-type protein differ, however, in their response to increases in pH. The ^1H NMR spectrum of huMIP-1 α broadens dramatically as the pH is raised above 4, while that of huMIP-1 α D26A is still relatively sharp at neutral pH, and higher (Ashfield, 1997). The broad spectral appearance is likely to be due to the formation of higher molecular weight aggregates. It was hoped that conditions could be found under which huMIP-1 α D26A would undergo slow exchange between various aggregates so that residues involved at the interfaces could be

identified in the NMR spectrum. Unfortunately, under all experimental conditions tested, exchange was still fast on the NMR chemical shift timescale. With the aim of slowing the exchange process, chemical modification of all of the lysine residues and the N-terminus was performed. The reductive alkylation of the ϵ -amino groups in lysine residues has been successfully employed to modify a number of proteins (Means, 1977; Geoghegan et al., 1979). The reaction proceeds via the formation of a Schiff base and is carried out under almost neutral conditions, conditions under which we have previously established that the protein is stable. The methyl source for the reaction was ^{13}C -labeled formaldehyde, and the reducing agent sodium cyanoborohydride, with nickel chloride to mop up free cyanide ions (Gidley & Sanders, 1982). Both mono- and di-methylated lysine residues may be produced and of course the N-terminal is also susceptible to mono- and di-methylation.

Three lysines are present in huMIP-1 α D26: K36, K44, and K60.

The first set of experiments to be performed were carried out with the protein (huMIP-1 α D26A) under conditions (pH, concentration, and buffer) in which dimer was known to be the dominant form (Patel et al., 1993). The modified protein arising from these was analyzed by high-performance liquid chromatography (HPLC) for purity and subsequently by MALDI-TOF mass spectrometry, analytical ultracentrifugation, and filter binding studies. These techniques established the integrity of the protein and the level and location of ^{13}C -methylation. Under the initial conditions, 2.5 mg/mL of protein with a reaction time of 48 h, and 25 °C, the protein was only partially modified. Peptide mapping was achieved by carboxamidomethylation to reduce the cysteine residues and denature the protein, followed by tryptic digestion. The procedure performed was identical to that used with nonmodified huMIP-1 α , and hence, the fragmentation analysis, also involving MALDI-TOF and HPLC, could be compared directly. It was deduced that K36 and K44 had been mono- and/or di-methylated. No methylation of the N-terminal or K60 was detected. A subsequent experiment was conducted under identical conditions but with a reaction time of 96 h. This resulted in complete dimethylation of K36, K44, and the N-terminal nitrogen. Importantly, again no methylation of K60 was observed. A further experiment was performed with a reaction time of 120 h, but again no methylation of K60 was observed. These results clearly suggested that in the dimer K36 and K44 are far more accessible to the solvent system than is K60. A model built for the dimeric form of huMIP-1 α , based on the solution structure of huMIP-1 β , in which the monomers are arranged in an elongated or cylindrical fashion, has the α -helix containing K60 a long way from the monomer–monomer interface and apparently solvent accessible. In contrast, K36 and K44 are somewhat buried in this model (Fig. 1A). However, if the monomers are arranged as a globular dimer, as adopted by the CXC chemokines, the reverse observation is made, and K60 is buried near the monomer interface (Fig. 1B). Therefore, our data clearly suggest a *globular* structure for huMIP-1 α D26A.

To probe this further, the reaction was repeated with the protein at higher dilution (0.25 mg/mL), and thus, with a greater proportion of monomer present. The outcome of this experiment was di-methylation of K36, K44, the N-terminus, and K60 (Fig. 2). This final experiment certainly supports the suggestion that K60 is not solvent accessible in the dimeric form of huMIP-1 α D26A.

The positioning of ^{13}C methyl labels is unambiguous following the mass spectral analysis of protein fragments. ^{13}C one- and two-dimensional heteronuclear single quantum coherence–nuclear Over-

Fig. 1. Ribbon diagram representing the structure of hu-MIP-1 α with monomers docked in (A) the cylindrical mode adopted by MIP-1 β (Lodi et al., 1994) and in (B) the globular mode adopted by IL-8 (Lodi et al., 1994). The illustration was produced using the program Swiss-Pdb Viewer, version 3.51 (1996) (N. Guex & M.C. Peitsch). The three lysines and the N-terminal serine are labeled with (A) and (B) referring to a particular monomer. It is clear that in the IL-8-like dimer, lysine 60 is buried.

hauser effect spectroscopy (HSQC-NOESY) NMR spectra were recorded for partially modified protein and a one-dimensional ^{13}C spectrum for the completely modified protein, enabling assignment of the various resonances. The HSQC-NOESY spectrum of the first sample displayed three sets of cross peaks (Fig. 3A); two for the di-methylated K36 and K44, and one, 10 ppm upfield (in the ^{13}C dimension) for mono-methylated K36 or K44 (Kalinowski et al., 1988) consistent with the mass spectrometric analysis. The HSQC-NOESY spectrum for the second sample (Fig. 3B) displayed two peaks at around 41.3 and 41 ppm (K36 and K44 di-methylated) and a third peak at ~ 40.5 ppm corresponding to the di-methylated N-terminal. The one-dimensional ^{13}C spectrum for the final, fully modified protein displayed five peaks (Fig. 4A). Three were at the chemical shifts observed for the methyls of K36, K44, and the N-terminal, and a further two either side of this envelope. The two final peaks are of almost half the intensity of the other peaks. According to the mass spectrometry results, these must arise from di-methylation of K60. We can rationalize the appearance of two peaks by considering the prochiral nature of the methyls on the ϵ -nitrogen. The nitrogen atom is prochiral if it is unable to invert (or inverts slowly on the chemical shift timescale). It is reasonable to assume that this would be the case if the lysine was in close proximity to other amino acid residues (as at an interface). This proposition is supported by the results of investigations of the concentration dependence of the ^{13}C NMR signals of the fully labeled protein. When the sample concentration in the NMR tube was reduced by a fivefold dilution (Fig. 4B), the ^{13}C spectrum displays an intense peak at the chemical shift of lysines 36/44 (overlapping with the N-terminal methyls) and two small satellite peaks due to K60 in the now low concentration of dimer.

The pattern of methylation detected via peptide mapping and mass spectrometric analysis and the NMR spectra recorded are all consistent with huMIP-1 α D26A existing as a globular dimer under the buffer conditions used here. It is clear from the high-resolution NMR studies performed previously that there is some flexibility in the monomer-dimer interactions for huMIP-1 α D26A (Czaplewski et al., 1999), hence making it difficult to determine the dimer structure to high resolution. However, the experiments reported here clearly point to a relatively long-lived dimer, and a dimer currently thought not typical of the CC chemokines. Another member of the β - or CC class of chemokines, MCP-3, has been observed as globular in the solution structure (Meunier et al., 1997), and the related protein, MCP-1, both globular and elongated dimers have been observed in a X-ray crystallographic studies (Lubkowski et al., 1997), so huMIP-1 α may not be a particular exception after all. More extensive studies would appear to be required before it may be established whether the subclasses of chemokines, CC and CXC, are structural as well as sequence subclasses.

Materials and methods: Synthetic huMIP-1 α and huMIP-1 α D26A were prepared from *Saccharomyces cerevisiae* as described previously (Czaplewski et al., 1999) and were supplied by British Biotech Pharmaceuticals Ltd. (Oxford, UK). HPLC purification of all protein samples followed protocols previously established at British Biotech Pharmaceuticals.

^{13}C -methyl labeling: For the first and second experiments, huMIP-1 α D26A was prepared to a concentration of 0.36 mM

Fig. 2. MALDI-TOF mass spectra of hu-MIP-1 α D26A. **A:** Modified at lysines 36 and 44 (mono and dimethylation present). **B:** Modified at lysine 36, 44, and the N-terminus. **C:** Modified at each lysine and the N-terminus. The calculated molecular mass of the fully modified protein is 7,788 Da (the added methyl groups were ^{13}C -labeled). The internal standard used for these measurements was bovine insulin (molecular weight 5,733.6 Da). The samples used here were the products of reactions carried out over 48 and 96 h for **A** and **B**, respectively, and of the reaction (following a 10-fold dilution of the protein) over 120 h for **C**.

(based on monomer molecular weight) (2.5 mg/mL) in HEPES buffer (50 mM) at pH 7.28. The reaction mixture was incubated at room temperature for 48 or 96 h in the presence of 1.2 mM ^{13}C -labeled formaldehyde. Formaldehyde was obtained by heating an aqueous solution of paraformaldehyde (CDN Isotopes, Croydon, UK) at 110 °C overnight in a sealed tube. The reaction mixture also contained 8 mM NaCNBH₃ and 2 mM NiCl₂ (Aldrich, Gillingham, UK). Following incubation the protein was dialysed successively (benzoylated tubing, 2,000 Da molecular weight cutoff, Sigma, Gillingham, UK) against water over a period of days. For the third experiment, the procedure was identical except that the protein concentration was 0.036 mM (0.25 mg/mL), and several reactions were run in parallel to obtain sufficient material for NMR studies.

Peptide mapping: For carboxamidomethylation samples of non-methylated, partially methylated, and fully methylated huMIP-1 α D26A (1.4 mM, 10 mg/mL) in PBS (Oxoid, Basingstoke, UK) were diluted (to 0.14 mM, 1 mg/mL) in buffer (urea, 8 M, TRIS, 0.35 M, EDTA 4 mM, pH 8.6). After addition of 1 M dithiothreitol

(Biorad, Hercules, California), the reaction mixture was incubated at 40 °C for 4 h. The mixture was allowed to cool to room temperature before addition of 1 M iodoacetamide (Sigma). The reaction mixture was incubated, in the dark, at room temperature for 30 min. The reaction was quenched by placing the reaction vessel on dry ice. Carboxamidomethylated protein was dialyzed successively against urea (2 M) and ammonium bicarbonate (0.1 M) at pH 8.0 for 60–70 h. Tryptic digestion of the resultant protein was achieved by diluting the protein in buffer (fourfold dilution) prior to addition of trypsin (theoretical activity 800 units/mL, Promega, Madison, Wisconsin) to give a theoretical activity of 17 units/mL. The reaction mixture was incubated at 30 °C for ~8 h. The reaction was quenched by standing on dry ice.

Mass spectrometry: The samples were prepared for mass spectrometry by loading 1 μ L of the matrix ACH (α -cyano 4-hydroxycinnamic acid, Thermo BioAnalysis) reconstituted in 70% acetonitrile, 0.1% trifluoroacetic to a concentration of 10 mg/mL, 0.2 μ L of the internal standard (bovine insulin MW 5,733.6 Da or human substance P MW 1,347.7 Da; 20 pmol, Sigma) and 0.5 μ L

Fig. 3. HSQC-NOESY spectra for hu-MIP-1 α D26A modified (A) at lysines 36 and 44 by the addition of one or two ^{13}C -labeled methyl groups at the ϵ -N, and (B) at lysine 36, 44, and the N-terminus by the addition of two ^{13}C -labeled methyl groups. The cross peak at 31 ppm (^{13}C dimension) in A is due to the presence of some monomethylation.

of the analyte sample (1–10 pmol) on to purpose-built slides (FinniganMAT). The slides were allowed to dry (2 to 5 min). Spectra were recorded on a Lasermat 200 Matrix Assisted Laser Desorption, time-of-flight mass analyzer. The data were acquired in high resolution and filter mode. Detected peaks were calibrated using the internal standard.

Sample preparation for NMR: Dry protein samples were dissolved in D_2O and lyophilized. The resultant powders were dissolved in 30% acetonitrile, 0.1% trifluoroacetic acid in D_2O solution. Samples volumes were 0.6 mL and sample concentrations varied between 0.012 and 4.8 mM for ^1H studies, and 0.24 and 4.8 mM for ^{13}C studies.

NMR spectroscopy: All one-dimensional spectra were acquired using a GE Omega 500. ^{13}C Spectra were recorded with Waltz-16 proton decoupling. Data were acquired in 32 K complex points over a spectral width of 50,000 Hz, and 8,000–16,000 acquisitions. FIDs were processed using FELIX version 2.30 (Biosym Technologies Inc., San Diego, California). A Gaussian function of -5 Hz line broadening and coefficient of 0.01 was used prior to Fourier transformation. Spectra were referenced to the carbon of the nitrile group of acetonitrile (at 117.7 ppm). The two-dimensional HSQC-NOESY (TPPI mode) spectra were recorded on a Bruker AMX500 spectrometer. The data were acquired over 256 t_1 s, each of 1,024 scans, in 2 K of data points. Data were processed using a Gaussian function in both dimensions (LB2 = -10 Hz, GB2 = 0.13, LB1 = -10 Hz, GB1 = 0.1) prior to Fourier transformation.

Fig. 4. 1D ^{13}C broad-band proton decoupled spectra for hu-MIP-1 α D26A modified at each lysine ϵ -N and the N-terminus by the addition of two ^{13}C labeled methyl groups at (A) 1.2 mM and (B) 0.24 mM protein concentration, in 30% acetonitrile, 0.1% trifluoroacetic acid in D_2O .

Supplementary material in Electronic Appendix: We have included MALDI-TOF mass spectra for nonmodified huMIP-1 α D26A, and for the protein modified at K36/K44, and modified at K36/K44 and the N-terminal to demonstrate that K60 could only be modified under conditions of high dilution.

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References

- Ashfield JT. 1997. Studies of multimerisation of huMIP-1 α [PhD Thesis]. Leeds, UK: University of Leeds.
- Baggiolini M, Dewald B, Moser B. 1994. Interleukin-8 and related chemotactic cytokines—CXC and CC chemokines. *Adv Immunol* 55:97–179.
- Bischoff SC, Krieger M, Brunner T, Rot A, von Tscharn V, Baggiolini M, Dahinden CA. 1993. RANTES and related chemokines activate human basophil granulocytes through different G-protein coupled receptors. *Eur J Immunol* 23:761–767.
- Chung C, Cooke RM, Proudfoot AEI, Wells TN. 1995. The three-dimensional solution structure of RANTES. *Biochemistry* 34:9307–9314.
- Clore GM, Appella E, Yamada M, Matsushima K, Gronenborn AM. 1990. 3-Dimensional structure of interleukin-8 in solution. *Biochemistry* 29:1689–1696.
- Clore GM, Gronenborn AM. 1991. Comparison of the solution nuclear magnetic resonance and crystal structure of interleukin-8—Possible implications for the mechanism of receptor binding. *J Mol Biol* 217:611–620.
- Covell DG, Smythers GW, Gronenborn AM, Clore GM. 1994. Analysis of hydrophobicity in the α -chemokine and β -chemokine families and its relevance to dimerization. *Protein Sci* 3:2064–2072.
- Creighton TE. 1993. *Proteins*, 2nd ed. New York: W.H. Freeman & Co.
- Czaplewski LG, McKeating J, Craven CJ, Higgins LD, Appay V, Brown A, Dudgeon T, Howard LA, Meyers T, Owen J, et al. 1999. Identification of amino acid residues critical for aggregation of CC chemokines MIP-1 α , MIP-1 β and RANTES: Characterisation of active disaggregated chemokine variants. *J Biol Chem* 274:16077–16084.
- Geoghegan KF, Ybarra DM, Feeney RE. 1979. Reversible reductive alkylation of amino groups in proteins. *Biochemistry* 18:5392–5399.
- Gidley MJ, Sanders JKM. 1982. Reductive methylation of proteins with sodium cyanoborohydride—Identification, suppression and possible uses of N-cyanomethyl by-products. *Biochem J* 203:331–334.
- Jentoft N, Dearborn DG. 1979. Labeling of proteins by reductive methylation using sodium cyanoborohydride. *J Biol Chem* 254:4359–4365.

- Kalinowski H-O, Berger S, Braun S. 1988. *Carbon-13 NMR spectroscopy*. Chichester, UK: J. Wiley & Sons.
- Laurence JS, Blanpain C, Burgner JW, Parmentier M, LiWang PJ. 2000. CC chemokine MIP-1 β can function as a monomer and depends on Phe13 for receptor binding. *Biochemistry* 39:3401–3409.
- Lipes MA, Napolitano M, Jeang KT, Chang NT, Leonard WJ. 1988. Identification, cloning and characterisation of an immune activation gene. *Proc Natl Acad Sci USA* 85:9704–9708.
- Lodi PJ, Garrett DS, Kuszewski J, Tsang ML-S, Weatherbee JA, Leonard WJ, Gronenborn AM, Clore GM. 1994. High-resolution solution structure of the β chemokine hMIP-1 β by multidimensional NMR. *Science* 263:1762–1767.
- Loetscher M, Gerber B, Loetscher P, Jones SA, Piali P, Clark-Lewis I, Baggiolini M, Moser B. 1996. Chemokine receptor specific for IP10 and mig: Structure, function, and expression in activated T-lymphocytes. *J Exp Med* 184:963–969.
- Lubkowski J, Bujacz G, Bogué L, Domaille PJ, Handel TM, Wlodawer A. 1997. The structure of MCP-1 in two crystal forms provides a rare example of variable quaternary interactions. *Nat Struct Biol* 4:64–69.
- Lundblad RL. 1995. *Techniques in protein modification*. Boca Raton, Florida: CRC Press Inc.
- Malkowski MG, Wu JY, Lazar JB, Johnson PH, Edward BFP. 1995. The crystal structure of recombinant human neutrophil-activation peptide-2 (M6L) at 1.9 Å resolution. *J Biol Chem* 270:7077–7087.
- Means GE. 1977. Reductive alkylation of amino groups. *Methods Enzymol XLVII*:469–478.
- Meunier S, Bernassau J-M, Guillemot J-C, Ferrara P, Darbon H. 1997. Determination of the three-dimensional structure of CC chemokine monocyte chemoattractant protein 3 by ^1H two-dimensional NMR spectroscopy. *Biochemistry* 36:4412–4422.
- Muller J. 1981. Stability of lactate dehydrogenase. 1. Chemical modification of lysines. *Biochim Biophys Acta* 669:210–215.
- Nieto MA, Palacian E. 1988. Structural changes of nucleosomal particles and isolated core-histone octaves induced by chemical modification of lysine residues. *Biochemistry* 27:5635–5640.
- Obaru K, Fukuda M, Maeda S, Shimada K. 1986. A cDNA clone used to study mRNA inducible in human tonsillar lymphocytes by a tumor promoter. *J Biochem* 99:885–894.
- Patel SR, Evans S, Dunne K, Knight GC, Morgan PJ, Varley PG, Craig S. 1993. Characterization of the quaternary structure and conformational properties of the human stem-cell inhibitor protein-LD78 in solution. *Biochemistry* 32:5466–5471.
- Proudfoot AEI. 1998. The chemokine family. Potential therapeutic targets from allergy to HIV infection. *Eur J Dermatol* 8:147–157.
- Rot A, Krieger M, Brunner T, Bischoff SC, Schall TJ, Dahinden CA. 1992. RANTES and macrophage inflammatory protein 1- α induce the migration and activation of normal human eosinophil granulocytes. *J Exp Med* 176:1489–1495.
- Royer WE Jr, Love WE, Fenderson FF. 1985. Co-operative dimeric and tetrameric clam hemoglobins are novel assemblages of myoglobin folds. *Nature* 316:277–280.
- Shaanan B, Lis H, Sharon N. 1991. Structure of a legume lectin with an ordered N-linked carbohydrate in complex with lactose. *Science* 254:862–866.
- St. Charles R, Waltz DA, Edwards BF. 1989. The three-dimensional structure of bovine PF4 at 3.0 resolution. *J Biol Chem* 264:2092–2099.
- Sticht H, Escher SE, Schweimer K, Forssmann W-G, Rosch P, Adermann K. 1999. Solution structure of human CC chemokine 2: A monomeric representative of the CC chemokine subtype. *Biochemistry* 38:5995–6002.
- Walz A, Peveri P, Aschauer H, Baggiolini M. 1987. Purification and amino acid sequencing of NAF, a novel neutrophil-activating factor produced by monocytes. *Biochem Biophys Res Commun* 149:755–761.
- Ward SG, Westwick J. 1998. Chemokines: Understanding their role in T-lymphocyte biology. *Biochem J* 333:457–570.
- Wolpe SD, Cerami A. 1989. Macrophage inflammatory proteins 1 and 2: Members of a novel superfamily of cytokines. *FASEB J* 3:2565–2573.
- Yamashita H, Nakatani H, Tonomura B. 1993. Change of substrate specificity by chemical modification of lysine residues of porcine pancreatic α -amylase. *Biochim Biophys Acta* 1202:129–134.
- Zhang MJ, Thulin E, Vogel HJ. 1994a. Reductive methylation and pKa determination of lysine sidechains in calbindin D-9k. *J Protein Chem* 13:527–535.
- Zhang XH, Chen LQ, Bancroft DP, Lai CK, Maione TE. 1994b. Crystal structure of recombinant human platelet factor-4. *Biochemistry* 33:8361–8366.