Human Recombinant Resistin Protein Displays a Tendency To Aggregate by Forming Intermolecular Disulfide Linkages†

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Received May 14, 2003; Revised Manuscript Received July 25, 2003

ABSTRACT: Resistin, a small cysteine rich protein secreted by adipocytes, has been proposed to be a link between obesity and type II diabetes by modulating the insulin signaling pathway and thus inducing insulin resistance. Resistin protein, with 11 cysteine residues, was not significantly homologous at the amino acid level to any other known cysteine rich proteins. Resistin cDNA derived from human subcutaneous adipose tissue was expressed in *Escherichia coli* as an N-terminal six-His-tag fusion protein. The overexpressed recombinant resistin was purified to homogeneity from inclusion bodies, after solubilization in 8 M urea, using a metal affinity column. While MALDI-TOF mass spectrometric analysis of the purified protein generated a single peak corresponding to the estimated size of 11.3 kDa, the protein exhibited a concentration-dependent oligomerization which is evident from size exclusion chromatography. The oligomeric structure was SDS-insensitive but β-mercaptoethanol-sensitive, pointing to the importance of disulfide linkages in resistin oligomerization. Estimation of free cysteine residues using the NBD-Cl assay revealed a concentration- and time-dependent increase in the extent of formation of disulfide linkages. The presence of intermolecular disulfide bond(s), crucial in maintaining the global conformation of resistin, was further evident from fluorescence emission spectra. Circular dichroism spectra revealed that recombinant resistin has a tendency to reversibly convert from α-helical to β-sheet structure as a direct function of protein concentration. Our novel observations on the biophysical and biochemical features of human resistin, particularly those shared with prion proteins, may have a bearing on its likely physiological function.

Diabetes, a major cause of morbidity and mortality, is a consequence of the loss of pancreatic β cell function, leading to a lack of insulin secretion and target tissue resistance to insulin. Insulin resistance and type 2 diabetes are caused by a combination of genetic and environmental factors. Of the known risk factors, namely, high-fat diet, insufficient exercise, and obesity, the latter is the most common and extensively studied. Although the connection between obesity and diabetes is not fully understood, several factors such as free fatty acids (FFA), TNF α, plasminogen-activated inhibitor-1 (PAI-1), leptin, resistin (*1*−*7*), and the recently described melanocortin 4 receptor (*8*, *9*) have been identified as the likely link. Resistin, a small cysteine rich polypeptide, was originally identified in mouse adipocytes (*6*). Resistin expression was induced during adipocyte differentiation and was shown to be downregulated by thiozolidenidiones (TZD). Circulating resistin levels were found to correlate with insulin resistance in both diet-induced and genetic models of obesity. Immunoneutralization of resistin with an anti-resistin antibody increased insulin sensitivity, whereas direct administration of resistin protein lowered the glucose tolerance threshold and impaired insulin activity (*6*). This discovery, which was an effort to establish a molecular link between obesity and type 2 diabetes, has raised several unanswered questions (*10*, *11*).

As opposed to the murine system, the association of human resistin with diabetes and obesity has been controversial. Several human studies have demonstrated the lack of a correlation between levels of resistin and obesity (*12*−*15*), whereas in others, an increased level of resistin was correlated with visceral obesity (*16*, *17*). Very recently, resistin mRNA levels in white adipose tissue and white blood cells were shown to increase upon treatment with lipopolysaccharide (*18*). The physiological role of resistin in diabetes and obesity can be better understood with knowledge of its molecular features. We previously showed (*19*) the presence of a large intron in the mouse resistin gene that is absent in humans. This intron carried a large number of

† B.A., A.K.S., and R.C. were supported by fellowships from the Council of Scientific and Industrial Research, New Delhi. S.G. thanks the Department of Biotechnology (DBT) for a postdoctoral fellowship. This work was supported by a grant to N.Z.E. from the Indian Council of Medical Research, New Delhi, and core support to CDFD from the DBT.

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10.1021/bi034782v CCC: $25.00
Published on Web 08/20/2003

transcription factor binding sites, including that of PPARγ. We and others further showed that the mouse resistin was downregulated in response to PPARγ and its agonist ciglitazone (*19*, *20*). We now provide data for the biophysical and biochemical characterization of human recombinant resistin protein. The recombinant resistin protein, overexpressed in *Escherichia coli*, was characterized by MALDI-TOF, circular dichroism, and fluorescence emission spectrometry. Our results point to the ability of resistin to oligomerize to high-molecular mass aggregates with inter- and intramolecular disulfide bond(s), which is important for maintaining the global conformation of the protein, with possible implications on its function.

MATERIALS AND METHODS

Cloning of the Human Resistin Gene. Human resistin was amplified by RT-PCR using total RNA isolated from subcutaneous adipose tissue. Upstream (5′-AG AGA TCT CTG TGC TCC ATG GAA GAA G-3′) and downstream (5′-GA GAA TTC GGG CTG CAC ACG ACA GCA-3′) oligonucleotide primers (the underlined bases denote the restriction sites, *Bgl*II and *Eco*RI, respectively) were synthesized (Microsynth). PCR was performed in a GeneAmp^R PCR system 9700 (Applied Biosystems) using an Access RT-PCR system (Promega Corp.) with the following cycles: 48 °C for 45 min, 94 °C for 2 min, followed by 10 cycles at 94 °C for 30 s, 52 °C for 30 s, and 68 °C for 30 s and 30 cycles at 94 °C for 30 s, 56 °C for 30 s, and 68 °C for 30 s, and finally 72 °C for 7 min. The amplified 282 bp cDNA fragment encoding the mature human resistin polypeptide (hRes) was ligated into *Bam*HI and *Hin*dIII sites of the pQE30 vector (Qiagen) to generate the recombinant plasmid pQEhRes carrying six histidines at the N-terminal end. The sequence of the resistin insert was confirmed by sequencing (ABI prism DNA sequencer).

Expression and Purification of Human Recombinant Resistin Protein. pQEhRes was transformed into competent *E. coli* M15 cells, and the transformants were grown at 37 °C in Terrific broth containing kanamycin (25 μg/mL) and ampicillin (100 μg/mL) antibiotics as selection markers. The cells were harvested, after induction with 1 mM IPTG, by centrifugation at 8000 rpm and 4 °C for 10 min, and the cell pellet was suspended in lysis buffer [50 mM sodium phosphate (pH 7.0) and 300 mM NaCl] containing 0.1 mg/mL lysozyme and incubated at 4 °C for 1 h. The cell suspension was further lysed by sonication using a probe sonicator (Branson) at 4 °C for 10 min. The lysed suspension was then centrifuged at 13 000 rpm for 30 min. The pellet, containing resistin inclusion bodies, was solubilized in lysis buffer containing 8 M urea. This was followed by centrifugation at 13 000 rpm for 30 min which yielded solubilized resistin. The supernatant was loaded onto a Talon column (Clontech), equilibrated under denaturing conditions with lysis buffer containing 8 M urea. The column was washed with lysis buffer containing 8 M urea and 5 mM imidazole to remove any nonspecifically bound protein. The recombinant resistin protein was eluted with lysis buffer containing 8 M urea and 150 mM imidazole. Fractions containing eluted protein were confirmed via analysis on 10% Tris-Tricene SDS−PAGE and finally pooled. The urea-solubilized resistin protein was reconstituted by dialyzing the pooled fractions against 10 mM Tris-HCl (pH 8.0). The dialysis buffer was changed thrice over a period of 9 h to obtain soluble resistin. The protein was concentrated using a 3 kDa MWCO Amicon filter (Millipore). Protein concentrations were estimated using Bradford reagent (*21*).

Spectrometric Analyses. Five picomoles of the purified human recombinant resistin protein was used for mass spectrometric analysis in a MALDI-TOF Voyager DE-STR mass spectrometer (Applied Biosystems) equipped with a 337 nm N2 laser. The mass spectrum was acquired with the reflector mode using α-cyanocinnamic acid as the matrix.

The intrinsic tryptophan fluorescence of resistin in various concentrations of urea and β-mercaptoethanol was measured in a Perkin-Elmer LS 3B spectrofluorimeter. Resistin (25 μg) in 2 mL of 10 mM Tris-HCl (pH 8.0) was incubated with 0−20% β-mercaptoethanol in the presence or absence of 8 M urea for 4 h prior to the spectra being recorded. The excitation wavelength was set at 295 nm, and the emission spectra were recorded in the wavelength range of 300−410 nm, at a scan speed of 280 nm/min. The bandwidth for both excitation and emission wavelengths was 10 nm. All the measurements were carried out at room temperature (RT).

The CD spectra of solutions containing various concentrations of resistin in 10 mM Tris-HCl (pH 8.0) either in the presence or in the absence of 8 M urea were recorded at RT in steps of 1 nm with 0.5 s averaging per point, and a 2 nm bandwidth in a spectropolarimeter (Jasco J-715) in the wavelength range of 190−250 nm. Spectra were signal averaged for four accumulations and baseline corrected by subtracting the spectra of the respective blank (*22*).

Estimation of Free Sulfhydryl Groups. Various concentrations of the protein were incubated at 37 °C for 1 h in the dark with an at least 100-fold excess of freshly prepared 7-chloro-4-nitrobenz-2-oxa-1,3-diazole (NBD-Cl) in DMSO. The resistin−Cys−S−NBD conjugate thus generated was quantitated by measuring the absorbance at 420 nm on a Unicam UV4 UV−visible spectrophotometer. The number of free SH groups in the protein was estimated from the calibration curve derived from the Cys−NBD-Cl conjugate after incubating L-cysteine with NBD-Cl under similar reaction conditions (*23*).

SDS−PAGE and Gel Filtration Chromatography. Protein samples were resolved on a 10% SDS−polyacrylamide gel using the Tris-Tricine buffer system. Purified resistin was treated with sample buffer in the absence or presence of varying concentrations of β-mercaptoethanol and dithiothreitol and subjected to SDS−PAGE and the gel stained with Coomassie R250.

Size exclusion chromatography was performed at RT using an FPLC system (Pharmacia Amersham) equipped with a Superdex-200 HR 10/30 column. To determine the molecular mass of the purified resistin protein, 150 μL each of 1.6 and 0.3 mg/mL resistin was loaded on the column at a flow rate of 0.5 mL/min and elution of the protein was monitored at 280 nm. The molecular masses of the respective peaks were determined on the basis of the elution volume of standard protein molecular mass markers supplied by Sigma.

RESULTS

Purification and Refolding of Human Recombinant Resistin. The 282 bp gene fragment encoding human resistin protein was amplified from human subcutaneous adipose

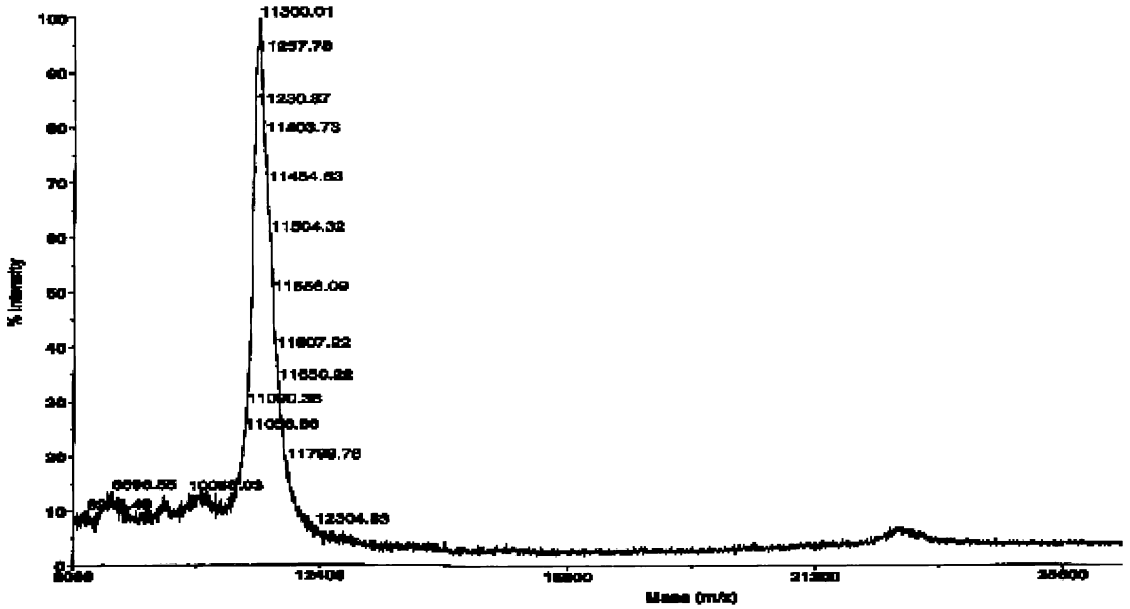

FIGURE 1: MALDI-TOF mass spectrometric analysis of purified human recombinant resistin reveals a mass of 11.3 kDa. Mass spectrometric analysis of 5 pmol of the purified human resistin was performed using a MALDI-TOF mass spectrometer. A major peak with an *m*/*z* value of 11.3 was obtained, confirming the purity of recombinant resistin.

tissue by RT-PCR and cloned for overexpression in *E. coli*. Purification of the protein was attempted from insoluble inclusion bodies solubilized in 8 M urea. The denatured protein was purified to apparent homogeneity by cobalt affinity chromatography and found to be more than 95% pure as seen via SDS−PAGE (data not shown). Solubilization of the protein was achieved by dialyzing against buffer without urea, and after several rounds of buffer changes, recombinant resistin could be obtained in a soluble form. MALDI-TOF mass spectrometric analysis of the purified protein yielded a peak with an *m*/*z* value of 11.3, which confirmed the purity of the protein, and was consistent with the predicted molecular mass of the human recombinant resistin (Figure 1).

Resistin contains two tryptophan residues at positions 81 and 98. Intrinsic tryptophan fluorescence emission spectra were recorded in the presence or absence of 8 M urea in an effort to assess the local environment of these tryptophan residues within the protein. The fluorescence emission maximum was observed at ~342 nm, suggesting that tryptophan residues are partially shielded from water in the folded protein. Moreover, the emission spectrum in the presence or absence of 8 M urea did not exhibit any shift in emission maxima (Figure 2; compare the black curve with the red one). This suggested that the tryptophan environment in resistin remained the same under both nondenaturing and denaturing conditions. The fluorescence scan was also recorded for refolded resistin in the presence of 5% β-mercaptoethanol, as a reducing agent, under nondenaturing and denaturing conditions. Once again, no shift in emission maxima could be observed (Figure 2; compare the green curve with the blue one). However, in the presence of 20% β-mercaptoethanol and 8 M urea, a significant shift toward a higher wavelength (red shift) was observed (Figure 2; compare the magenta curve with the black one). These results indicate that cysteine residues involved in disulfide bond formation are stabilizing the native structure even in the presence of 8 M urea, and the protein can be completely unfolded under only strong reducing conditions.

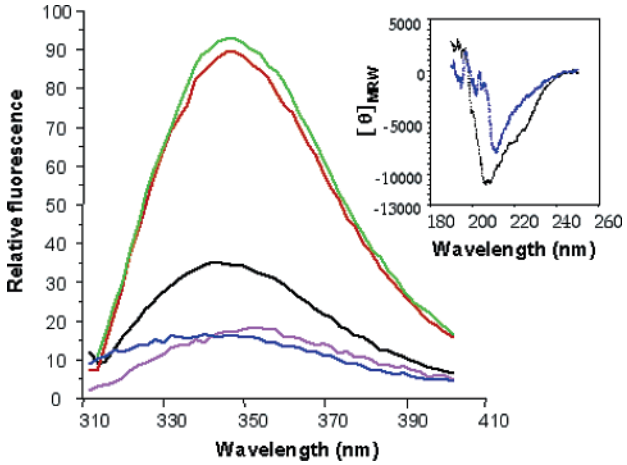

FIGURE 2: Resistin is resistant to denaturation by urea which is evident from fluorescence emission and CD spectra. The resistin protein was excited at a wavelength of 295 nm, and fluorescence emission spectra were recorded in the range of 300−410 nm under different conditions. The different spectra are for native resistin (black line), resistin denatured with 8 M urea (red line), resistin reduced with 5% β-mercaptoethanol (green line), resistin reduced with 5% β-mercaptoethanol with 8 M urea (blue line), and resistin reduced with 20% β-mercaptoethanol with 8 M urea (magenta line). In the inset are shown far-UV CD spectra of purified resistin protein recorded in the presence and absence of 8 M urea in the wavelength range of 190−250 nm. The molar ellipticity, $[\theta]_{MRW}$, was plotted as a function of wavelength. Note that the CD spectra of human resistin protein recorded in 10 mM Tris-HCl (pH 8.0) (black line) show predominantly α-helical structure similar to that in the presence of 8 M urea (blue line).

These results were further strengthened by recording CD spectra of recombinant resistin in the presence and absence of 8 M urea. It was noticed that the spectra of 1.2 mg/mL recombinant resistin in the presence and absence of urea are essentially similar and predominantly represented α-helical secondary structure (Figure 2, inset). These results indicate that the recombinant resistin protein retained its secondary structure even in 8 M urea, and this unusual behavior can probably be attributed to inter- and intramolecular disulfide

Table 1: Free Sulfhydryl Group Estimation[a]

sulfur	protein concentration at which the assay was performed (mg/mL)	time point at which the assay was performed	no. of free sulfhydryl groups detected
1	0.3	freshly prepared protein	10.3 ± 0.18
2	0.17	2-day-old protein	9.2 ± 0.19
3	1.0	2-day-old protein	2.7 ± 0.24
4	1.0	5-day-old protein	2.9 ± 0.24

[a] The number of free sulfhydryl groups was estimated as a function of protein concentration at various time points using the NBD-Cl reagent. The protein−NBD-Cl adduct which has an absorption maximum at 420 nm was assessed. The number of free SH groups was determined by extrapolation from a standard graph made by estimating the number of L-cysteines. Note that the number of free SH groups decreased to three as the concentration and the age of the protein increased.

bond formation within the resistin protein. The protein therefore appears to be resistant to urea denaturation.

Human resistin has 11 cysteine residues which might partially be responsible for conferring an unusual stability to urea denaturation through intra- and intermolecular disulfide linkages. The number of free SH groups of proteins can be estimated from their reaction with NBD-Cl to form an adduct which can be measured at 420 nm in a spectrophotometer (*23*). Estimation of the number of free SH groups in resistin showed that fresh, but dilute, protein has 10 free cysteines (Table 1), whereas the eleventh might be involved in formation of a disulfide bond between two monomers. This suggests that human recombinant resistin is a dimer linked through an intermonomer disulfide bond involving a cysteine residue, as reported previously (*24*). However, upon concentration and storage, the free cysteine residues tend to be involved in disulfide bond formation as detected by the decrease in the number of free sulfhydryl groups. These disulfide bonds could likely lead to aggregation of the protein.

SDS−PAGE under reducing and nonreducing conditions indeed demonstrated the SH-dependent oligomeric nature of resistin. In the absence of DTT as a reducing agent, it was found that purified resistin has a mixed population of monomer, dimer, trimer, tetramer, etc., although monomeric resistin constituted the major population. When resistin was incubated with 100 mM DTT, mostly monomeric and some dimeric species could be observed on SDS−PAGE. Moreover, increasing the concentration of DTT to 500 mM did not result in further reduction of dimeric species to a monomeric form (data not shown). However, when β-mercaptoethanol was used as a reducing agent at a concentration ranging from 5 to 30% (Figure 3), a concentration-dependent reduction to resistin monomers was evident. A single protein band corresponding to the monomeric form could be seen when resistin was incubated with 30% β-mercaptoethanol. These experiments, therefore, suggested that resistin has intersubunit disulfide bonds which could be completely reduced by only a high concentration of β-mercaptoethanol.

Structural Analyses of Human Recombinant Resistin. To determine if the purified human resistin protein possessed specific secondary structure, the far-UV CD spectrum of the protein was recorded (Figure 4) at various protein concentrations. These spectra demonstrated that resistin at a concentration of up to 1.2 mg/mL displays mainly α-helical structures; however, at a concentration of >1.8 mg/mL, it was found

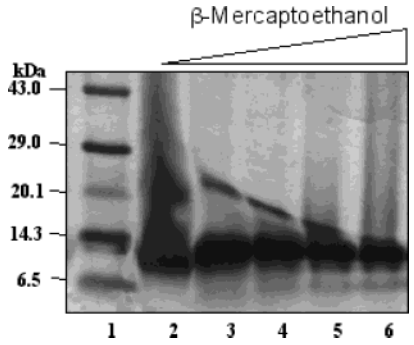

FIGURE 3: Resistin is reduced to a monomeric form in the presence of a high concentration of β-mercaptoethanol. SDS−PAGE of resistin in the presence of increasing concentrations of β-mercaptoethanol: lane 1, molecular mass markers; lane 2, nonreduced resistin protein; and lanes 3−6, resistin protein in the presence of 5, 10, 20, and 30% β-mercaptoethanol, respectively.

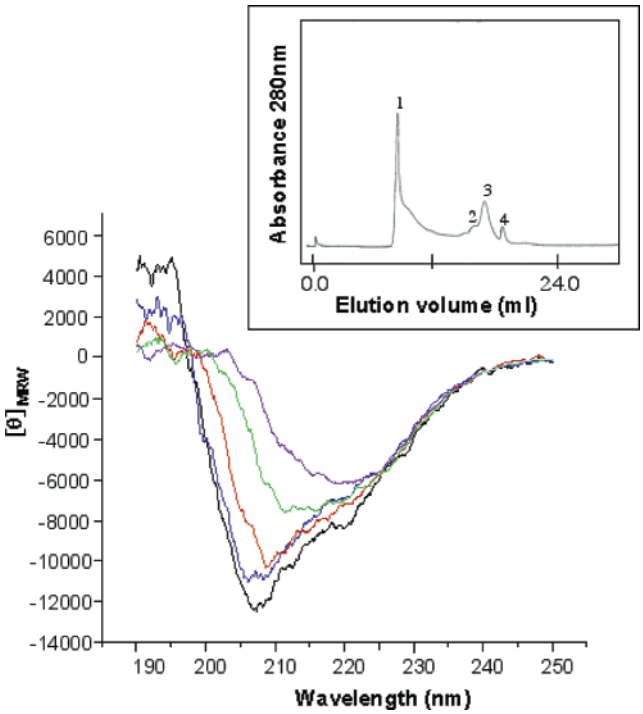

FIGURE 4: Resistin undergoes a concentration-dependent α- to β-conformational change. The CD spectra of human resistin protein were recorded from 190 to 250 nm at increasing protein concentrations in 10 mM Tris-HCl (pH 8.0), and the molar ellipticity was plotted vs wavelength. The thin black line is the spectrum of human resistin at 0.6 mg/mL protein, blue line 0.8 mg/mL, orange line 1.2 mg/mL, green line 1.8 mg/mL, and violet line 2.4 mg/mL. In the inset is a gel filtration profile of 1.6 mg/mL purified human resistin which reveals a higher-order oligomeric structure. Peak 1 corresponds to the protein fraction eluted in the void volume. Peak 2 corresponds to an oligomer of ~90−100 kDa. Peak 3 corresponds to 40−50 kDa. Peak 4 corresponds to ~25 kDa protein.

to exist as a mixed population of α-helix and β-sheet structure. Furthermore, when the ellipticity of 2.4 mg/mL resistin protein was measured as a function of wavelength, the protein exhibited mainly β-sheet structure which became more pronounced at a still higher concentration (4.5 mg/mL, data not shown). Furthermore, sequential dilution of 4.5 mg/mL protein to 2.4, 1.8, 1.2, 0.8, and 0.6 mg/mL showed the reversal of secondary structure from β-sheet to α-helix (data not shown).

To analyze the quaternary structure organization of recombinant resistin, size exclusion chromatography was

used. The gel filtration chromatogram at a concentration of 1.6 mg/mL on a Superdex 200 column revealed that a major fraction of the loaded protein formed a large oligomer as the major peak (Figure 4, inset, peak 1) that eluted in the void volume fraction. This was followed by peaks 2−4, corresponding to ∼90−100, ∼40−50, and ∼25 kDa, respectively. The gel filtration profile of 0.3 mg/mL protein showed a higher percentage of the 25 kDa form than of the 1.6 mg/mL protein. This once again suggested that recombinant resistin exhibited a tendency to aggregate as the protein concentration increased.

DISCUSSION

Resistin cDNA was amplified from human subcutaneous adipose tissue and expressed in *E. coli*, and recombinant resistin was purified to homogeneity from inclusion bodies under denaturing conditions to generate an 11.3 kDa protein which is evident from MALDI-TOF mass spectrum analysis. The presence of two tryptophan residues in the protein (at positions 81 and 98) was exploited to evaluate the folded state. This was analyzed by recording the intrinsic tryptophan fluorescence of the protein in the presence and absence of 8 M urea. Resistin exhibited an emission maximum at 342 nm, indicating that the environment of the two tryptophans is partially polar. No significant red shift in the emission maxima, a characteristic of unfolded proteins, was observed in the presence of 8 M urea. Secondary structure analysis by circular dichroism also showed that there was no loss of secondary structure even in the presence of 8 M urea. The dye 1-anilinonaphthalene-8-sulfonic acid (ANS), which fluoresces in a nonpolar environment, was used to determine the hydrophobicity of human resistin. Expectedly, ANS did not exhibit (data not shown) any fluorescence in the presence of resistin indicating the absence of surface hydrophobic patches, thereby complementing the above observations.

Human resistin contains 11 cysteine residues that are also highly conserved in other protein members of the resistin family (*26*). The involvement of cysteine residues in disulfide linkages or the availability of free cysteine residues was determined with the NBD-Cl assay, which is based on the ability of the latter to form an adduct with free cysteine. Estimation of the number of free SH groups with the NBD-Cl reagent showed that freshly purified, dilute human resistin has 10 free cysteine residues with one cysteine involved in intermolecular disulfide bond formation. It was earlier shown that cysteine 26, the first cysteine residue at the N-terminal end, of the mouse resistin and mouse RELMβ (a member of the resistin family of proteins) was involved in an intermolecular disulfide linkage, generating a homodimer (*24*, *25*). Results given in Table 1 additionally show that as a function of protein concentration and/or storage time, the number of free cysteine residues tends to decrease, indicating that the protein stabilizes itself by forming either inter- or intramolecular disulfide bonds. With 10 free cysteine residues in a freshly purified dilute protein in the beginning, the number of free SH groups decreased to three as the protein was concentrated. This oligomerization could be due to disulfide linkages between the subunit molecules and is supported by our observation that disruption of all disulfide linkages by more than 20% β-mercaptoethanol leads to protein precipitation. To further confirm the involvement of cysteine residues in disulfide bond formation, reduced and nonreduced resistin proteins were analyzed via SDS−PAGE. The nonreduced protein exhibited higher-molecular mass protein bands corresponding to dimer, trimer, and tetramer, in addition to the monomeric form of resistin. Reduction of the protein with 5% β-mercaptoethanol could not abolish the dimeric species; the same, however, was accomplished with only 30% β-mercaptoethanol. DTT at a concentration of 0.5 M was surprisingly ineffective at reducing the protein completely.

To dissect the importance of inter- and intramolecular disulfide bonds in maintaining the higher-order oligomeric structure, the tryptophan fluorescence was recorded in the presence and absence of 8 M urea and β-mercaptoethanol as the reducing agent. The emission maximum, at a low concentration (5%) of the reducing agent, was similar to that of the protein in the presence of 8 M urea. However, a high concentration (20%) of β-mercaptoethanol in the presence of 8 M urea completely unfolds the protein, as is evident from the shift in the emission maximum from 342 nm for the folded protein to 352 nm. The attempt to record far-UV CD and fluorescence spectra in the presence of 20% β-mercaptoethanol failed due to a visible precipitation of the protein. These results suggest that the disulfide bonds might play a crucial role in maintaining the folded and stable conformation of resistin.

Far-UV CD spectra of purified human resistin protein were recorded at various protein concentrations to determine the effect on secondary structure. Assessment of secondary structure by far-UV CD revealed yet another interesting structural feature of resistin. At a low concentration of the protein, it possessed a predominantly α-helical structure. However, as the concentration of the protein was increased, a significant shift toward β-conformation was observed. The concentration-dependent conformational change is reminiscent of many proteins that exert their functional properties through such a conformational switch. Examples such as prions, amyloids, etc., also exhibit a tendency to aggregate in the β-conformation. Resistin, however, differs from these proteins in terms of its ability to reverse the aggregation upon dilution. The disease manifestations can thus be attributed to α- to β-conformational change, followed by protein aggregation.

Whether the aggregation of resistin is mediated by conformational change or through formation of multiple disulfide bonds remains to be ascertained. We have confirmed that only one of the 11 cysteine residues in a fresh dilute preparation of resistin is involved in a disulfide linkage. Under oxidative conditions, the other 10 cysteine residues may form disulfide linkages depending on their surface availability, leading to formation of aggregates. A member of the resistin family, RELMα from mouse, involved in adipocyte differentiation has been similarly shown to undergo disulfide-linked homo-oligomerization (*27*). Thus, resistin aggregation, conformational changes, and multiple disulfide cross-linkages may appear to be interrelated. While further studies, such as site-directed mutagenesis, to dissect these folding phenomena are underway, the intrinsic tendency of resistin to aggregate as suggested by gel permeation chromatography, and the concentration-dependent change in its secondary structure, are suggestive of resistin having a different functional role in the disease processes depending on its structure. It is therefore conceivable that the level of

expression of resistin gene will then modulate the higher-order structure of resistin and by implication its function.

ACKNOWLEDGMENT

We acknowledge Drs. Ch. Mohan Rao and P. Raghu for critical discussions during the course of this study. We thank Drs. Ghafoorunissa and B. Sivakumar for their support.

REFERENCES

1. Boden, G. (1997*) Diabetes 46*, 1−10.
2. Hotamisligil, G. S. (1999) *J. Int. Med. 245*, 621−625.
3. Steppan, C. M., and Lazar, M. A. (2002) *Trends Endocrinol. Metab. 13*, 18−23.
4. Wiman, B., Chmielewska, J., and Ranby, M. (1984) *J. Biol. Chem. 259*, 3644−3647.
5. Friedman, J. M. (2000) *Nature 404*, 632−634.
6. Steppan, C. M., Bailey, S. T., Bhat, S., Brown, E. J., Banerjee, R. R., Wright, C. M., Patel, H. R., Ahima, R. S., and Lazar, M. A. (2001) *Nature 409*, 307−312.
7. Banerjee, R. R., and Lazar, M. A. (2003) *J. Mol. Med. 81*, 218−226.
8. Farooqi, I. S., Keogh, J. M., Yeo, G. S., Lank, E. J., Cheetham, T., and O'Rahilly, S. (2003) *N. Engl. J. Med. 348*, 1085−1095.
9. Branson, R., Potoczna, N., Kral, J. G., Lentes, K. U., Hoehe, M. R., and Horber, F. F. (2003) *N. Engl. J. Med. 348*, 1096−1103.
10. Ehtesham, N. Z. (2001) *Curr. Sci. 80*, 1369−1371.
11. Ehtesham, N. Z. (2002) *Curr. Sci. 83*, 1190−1191.
12. Nagaev, I., and Smith, U. (2001) *Biochem. Biophys. Res. Commun. 285*, 561−564.
13. Savage, D. B., Sewter, C. P., Klenk, E. S., Segal, D. G., Vidal-Puig, A., Considine, R. V., and O'Rahilly, S. (2001) *Diabetes 50*, 2199−2202.
14. Janke, J., Engeli, S., Gorzelniak, K., Luft, F. C., and Sharma, A. M. (2002) *Obes. Res. 10*, 1−5.
15. Kielstein, J. T., Becker, B., Graf, S., Brabont, G., Haller, H., and Fliser, D. (2003) *Am. J. Kidney Dis. 42*, 62−66.
16. McTernan, P. G., McTernan, C. L., Chetty, R., Jenner, K., Fisher, F. M., Lauer, M. N., Crocker, J., Barnett, A. H., and Kumar, S. (2002) *J. Clin. Endocrinol. Metab. 87*, 2407.
17. McTernan, C. L., McTernan, P. G., Harte, A. L., Levick, P. L., Barnett, A. H., and Kumar, S. (2002) *Lancet 359*, 46−47.
18. Lu, S. C., Shieh, W. Y., Chen, C. Y., Hsu, S. C., and Chen, H. L. (2002) *FEBS Lett. 530*, 158−162.
19. Ghosh, S., Singh, A. K., Aruna, B., Mukhopadhyay, S., and Ehtesham, N. Z. (2003) *Gene 305*, 27−34.
20. Song, H., Shojima, N., Sakoda, H., Ogihara, T., Fujishiro, M., Katagiri, H., Anai, M., Onishi, Y., Ono, H., Inukai, K., Fukushima, Y., Kikuchi, M., Shimano, H., Yamada, N., Oka, Y., and Asano, T. (2002) *Biochem. Biophys. Res. Commun. 299*, 291−298.
21. Bradford, M. M. (1976) *Anal. Biochem. 72*, 248−254.
22. Alder, A. J., Greenfield, N. J., and Fasman, G. D. (1973) *Methods Enzymol. 27*, 675−735.
23. Birkett, D. J., Price, N. C., Radda, G. K., and Salmon, A. G. (1970) *FEBS Lett. 6*, 346−348.
24. Banerjee, R. R., and Lazar, M. A. (2001) *J. Biol. Chem. 276*, 25970−25973.
25. Juan, C.-C., Kan, L.-S., Huang, C.-C., Chen, S.-S., Ho, L.-T., and Au, L.-C. (2003) *J. Biotechnol. 103*, 113−117.
26. Steppan, C. M., Brown, E. J., Wright, C. M., Bhat, S., Banerjee, R. R., Dai, C. Y., Enders, G. H., Silberg, D. G., Wen, X., Wu, G. D., and Lazar, M. A. (2001) *Proc. Natl. Acad. Sci. U.S.A. 98*, 502−506.
27. Blagoev, B., Kratchmarova, I., Nielsen, M. M., Fernandez, M. M., Voldby, J., Andersen, J. S., Kristiansen, K., Pandey, A., and Mann, M. (2002) *J. Biol. Chem. 277*, 42011−42016.

BI034782V