

## Mutant proteins of human interleukin 2

### Renaturation yield, proliferative activity and receptor binding

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(Received October 17/December 13, 1988) – EJB 88 1221

Mutants, i.e. proteins altered by mutation of their genes, of interleukin 2 (IL2) were generated by oligonucleotide-directed mutagenesis *in vitro*. All acidic and basic residues conserved between man and mouse were exchanged as well as four lipophilic residues contained within four hydrophobic segments of the protein. The mutants were produced in *Escherichia coli* and submitted to a renaturation and purification protocol, before bioactivity and receptor binding of each of them was determined.

All mutants besides two (K44/T125 and Q110/T125) could be renatured and purified. One mutant (K94/T125) exhibited a more than tenfold-improved renaturation yield.

One amino exchange (Asp-20 to Asn) resulted in an about 20-fold reduction in proliferative activity and high-affinity receptor binding. The binding to the low-affinity IL2-binding protein (Tac antigen) was unimpaired.

A second exchange (Arg-38 to Gln) had no effect on proliferative activity. The binding to both the high- and the low-affinity receptor, however, was reduced about 20-fold.

Preliminary trials on the stability of these mutants by guanidinium hydrochloride denaturation studies detected no differences between wild-type interleukin 2 and mutants.

It is suggested that Asp-20 forms part of the binding site for the large receptor subunit whereas Arg-38 is involved in the contact site to the small subunit.

The transient synthesis of interleukin 2 (IL2) and its receptor by activated T lymphocytes apparently represents an essential step in the elaboration of the cellular immune response (for recent reviews see [1–3]). The binding of IL2 to a high-affinity receptor ( $K_d \approx 10$  pM) provides the growth-signal leading to the clonal expansion of antigen-specific T cells.

Interestingly, the high-affinity receptor has been established to be assembled from a low-affinity ( $K_d \approx 10$  nM) IL2-binding protein of  $M_r$  55 000 (Tac antigen,  $\beta$  subunit) and an intermediate-affinity ( $K_d \approx 1$  nM) receptor protein of  $M_r$  70 000–75 000 ( $\alpha$  subunit) that is likely to represent the growth-signal-transducing moiety [4, 5]. The human IL2 secreted by activated T cells is a 133-residue glycoprotein [6, 7]. The cDNA has been cloned [8, 9] and could be efficiently expressed in *Escherichia coli*. The recombinant IL2 when properly renatured and purified exhibits a specific biological activity as high as the authentic T cell product. The availability of chemical quantities of recombinant IL2 paved the way for studies on its potential use as immunotherapeutic agent (see e.g. [10]) and for studies on its biochemical properties. The three-dimensional structure at 0.3-nm resolution has been described recently [11].

Several previous communications have addressed the question of receptor-binding site(s) on the IL2 protein by using immunological [12–14] or genetic techniques [15–18]. The analyses of mutant proteins (mutants) of IL2 remained to a

large extent preliminary, however, since the proteins were not renatured and purified before measuring proliferative or receptor-binding activity. Thus, effects of a certain mutation on the folding or on the receptor-binding sites could not be clearly differentiated.

During the studies presented in this paper a series of charged residue positions and of hydrophobic positions were exchanged which cover more or less the whole surface of IL2. The mutants were all submitted to a renaturation and purification protocol before analysing biological activities. Two mutants appeared which define positions involved in high- or in low-affinity receptor binding. Surprisingly, one mutant was obtained exhibiting a large increase in yield during renaturation.

## METHODS

### Construction of mutant strains

A cDNA coding for the mature part of human IL2 [8] plus an initiator methionine [19] was submitted to oligonucleotide-directed mutagenesis by the gapped duplex DNA approach [20, 21]. The synthetic oligonucleotides comprised 6–16 residues before and after the nucleotide(s) to be exchanged, and they were provided by Dr H. Blöcker (GBF, Braunschweig) or synthesised on a DNA synthesiser (Applied Biosystems, model 380). Mutations in the Thr-125 background were constructed by starting with a cDNA containing an ACT codon in place of the TGT coding for Cys-125 of wild-type IL2. Each mutation was detected by DNA sequence analysis of single-stranded bacteriophage DNA.

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Abbreviations. IL2, interleukin 2; mutant, protein altered by mutation of the gene.

The mutated cDNA was excised as an *NcoI*–*Bam*HI fragment from the double-stranded viral DNA and recombined with a temperature-regulated expression vector pR<sup>TS</sup>pRC109 which is similar to P1LA 502 [22] with the exception that the lambda leftward promoter and the polylinker are missing. A *recA*<sup>-</sup> derivative of *E. coli* strain JM103 [23] was used as host. The integrated I12 cDNA was sequenced and, when the mutation had been confirmed, the strain was used for the expression of the mutein.

#### Isolation of I12 muteins

An *E. coli* strain exhibiting temperature-regulated expression of a mutein was grown at 30°C in 0.8 l Luria broth plus ampicillin (50 µg/ml) to an *A*<sub>550</sub> of 0.5. The 2-l flasks were then immediately transferred to a rotatory water bath and incubated at 42°C for another 2–3 h at 200 rotations/min to an *A*<sub>550</sub> of 1.2–1.5. The cells (1.5–2 g wet mass) were harvested by centrifugation for 10 min at 6000 × *g*, washed once with 50 mM Tris/HCl, pH 8, and frozen at –20°C.

The recombinant I12 muteins were renatured and purified according to Kato et al. [24] with some modifications. Briefly, cells dispersed in 10 vol. (vol./wet mass) sucrose/Tris/EDTA buffer (0.375 M sucrose, 10 mM Tris/HCl pH 8, 1 mM EDTA) were sonicated five times for 1 min at 300 W in an ice bath. The particulate fraction containing inclusion bodies of the highly expressed mutein was sedimented at 17000 × *g* for 20 min at 4°C. The supernatant and a greenish fluffy layer were discarded. The tight white sediment was washed by resuspension and centrifugation once with sucrose/Tris/EDTA buffer and twice with Tris/EDTA buffer (50 mM Tris/HCl pH 8, 1 mM EDTA). The final sediment (0.3–0.4 g wet mass) was suspended in 10 vol. 0.1 M Tris/HCl, pH 8, yielding about 20 mg protein/ml.

The protein was dissolved by adding 3 vol. 8 M guanidinium chloride in 0.1 M Tris/HCl, pH 8, and 0.1% (by vol.) 2-mercaptoethanol. After incubation for 2 h at ambient temperature, the mixture was clarified by a 15-min centrifugation at 17000 × *g* also at ambient temperature. The resultant solution, containing about 5 mg protein/ml, was diluted fivefold with ice-cold water, and was then dialysed for 16–20 h at 4°C against 20 vol. 10 mM Tris/HCl, pH 8, 1 mM EDTA. The very cloudy suspension was adjusted to pH 5 with acetic acid and centrifuged for 20 min at 17000 × *g*.

The supernatant was pumped over 2 ml CM-Sepharose CL-6B (Pharmacia) equilibrated with 25 mM ammonium acetate, pH 5. The adsorbed I12 was eluted with a linear gradient of 0–0.5 M NaCl in 25 mM ammonium acetate, pH 5.

The eluted I12-containing fractions were pooled and submitted directly to HPLC over a C<sub>4</sub> column (Vydac 214 TP54). The column was developed with a gradient of 28–70% acetonitrile in 0.1% trifluoroacetic acid in water. The eluted protein was pooled and stored as aliquots at –20°C.

#### Preparations of <sup>3</sup>H-labeled I12

Mutein T125 was mass-labeled with <sup>3</sup>H-labeled amino acids (TRK 550, Amersham) and isolated as described above with the following modifications. Cells were grown in 10 ml Vogel Bonner minimal medium plus 50 µg ampicillin/ml. <sup>3</sup>H-labeled amino acids were added (2.5 mCi in 250 µl water) 3 min after transfer of the culture to 42°C. Cells were harvested 30–60 min later. Inclusion bodies were dissolved in 500 µl 6 M guanidinium hydrochloride. Omitting the CM-Sepharose

chromatography, the supernatant after dialysis was mixed with an equal volume of 0.1% trifluoroacetic acid, centrifuged, and the supernatant was then submitted immediately to HPLC. The amount of I12 (0.9–6 µg) was estimated by a proliferation assay and determined by competition with unlabeled I12 in a receptor-binding assay. Specific radioactivities obtained ranged over 100–360 nCi/pmol I12 (6.7–24 µCi/µg I12).

#### Proliferation assays

The bioactivity of I12 and I12 muteins was estimated by measuring [<sup>3</sup>H]thymidine incorporation into an I12-dependent murine T cell line [25] (CTLL-2; ATCC MB214) or concanavalin-A-activated human peripheral blood cells [26] (Blutbank des Deutschen Roten Kreuzes, Würzburg). The assays were calibrated with the I12 standard from the Biological Response Modifier Program (NIH, Bethesda). 1 unit I12/ml gave a half-maximal response in the murine CTLL assay.

#### Receptor binding

For high-affinity binding 2 × 10<sup>6</sup> HUT 102 (ATCC TIB162) cells were incubated for 1 h at 4°C with 250 pM <sup>3</sup>H-labeled I12 (mutein T125) plus serial dilutions of unlabeled muteins from 0.05 pM to 50 nM. Bound radioactivity was measured after washing twice with ice-cold incubation medium (RPMI plus 10% fetal calf serum) by resuspension and centrifugation for 10 s at 7000 × *g*.

For low-affinity binding [27], 10<sup>6</sup> HUT 102 cells were incubated for 1 h at 4°C with 10 nM <sup>3</sup>H-labeled I12 (mutein T125) plus serial dilutions of unlabeled muteins from 1 nM to 5 µM. Cells were immediately centrifuged through a silicon layer for determination of bound I12. Sedimented cells were suspended in 0.1 ml incubation buffer, mixed with 0.1 ml 2% SDS in the scintillation vial before addition of 3 ml emulsifier Scintillator 299<sup>TM</sup> (Packard).

#### Guanidinium hydrochloride denaturation curves

HPLC-purified I12 muteins were lyophilised and dissolved at a concentration of 10 µM (= 150 µg/ml) in 25 mM ammonium acetate, pH 5. The protein was then diluted to 1 µM to achieve the different guanidinium chloride concentrations (0–7.2 M) in the pH-5 buffer. Fluorescence emission spectra were recorded between 300–450 nm during excitation at 290 nm (fluorescence spectrophotometer, Schoeffel model RRS 1000).

#### Analytical procedures

Protein was determined in the inclusion bodies by the Lowry method [28] after precipitation with trichloroacetic acid in the presence of deoxycholate. Protein of purified I12 muteins was measured by absorbance at 280 nm using an absorption coefficient of 1.22 × 10<sup>4</sup> M<sup>-1</sup> cm<sup>-1</sup> (1 mg I12/ml yields an absorbance at 280 nm of 0.8). Proteins dissolved in dodecyl sulfate were separated by electrophoresis on 12% polyacrylamide gels according to [29].

## RESULTS

#### Purification and renaturation of recombinant I12 muteins

The human recombinant I12 after heat induction constituted 25–30% of the total *E. coli* protein. It was quantita-



Fig. 1. Purification of recombinant human IL2 from *E. coli*. The various protein fractions were submitted to SDS gel electrophoresis and stained with Coomassie blue. Lanes show protein (a) of whole cells, (b) of supernatant, (c) of particulate fraction (= inclusion bodies), (d) after CM-Sepharose chromatography, and (e) after HPLC

tively recovered in the particulate fraction of the broken cells (inclusion bodies) contaminated to less than 10% by other proteins. The protein was dissolved completely in 6 M guanidinium chloride. After the renaturation step, however, only a small percentage was recovered in soluble form. Minor amounts of contaminating proteins and other ultraviolet-absorbing substances were removed and the renatured IL2 was concentrated by CM-Sepharose chromatography at pH 5. The final purification was achieved by HPLC. The gel electrophoretic analyses of the various fractions obtained during a representative purification are shown in Fig. 1.

In Table 1 the amino acid exchanges of the constructed and analysed muteins are compiled, together with the location in helices A–F or in helix-connecting loops according to the three-dimensional structure [11]. Most of these muteins could be renatured and purified in yields comparable to the wild type, i.e. from 100 mg protein of inclusion bodies 0.8–2.4 mg protein of the finally purified muteins were obtained (Table 2). It has not been analysed whether this range of differences in yield was caused by specific properties of certain muteins or by experimentation.

For some muteins, however, much more pronounced deviations in renaturation yield were observed. The Phe-44 to Lys or the Glu-110 to Gln exchange completely prevented renaturation under the conditions applied. On the other hand, the K94/T125 mutein was recovered with a final yield of about 25% based on the protein content of the inclusion bodies (Table 2). It is worth noting that, if the guanidinium-chloride-dissolved muteins were submitted to a proliferation assay, extremely low specific activities were observed for the two non-renaturing muteins ( $< 10^3$  U/mg) whereas a maximal specific activity of  $2-4 \times 10^7$  U/mg protein was observed for the super-renaturing K94/T125 mutein.

Table 1. Muteins of human interleukin 2

For designation of the muteins, the amino acid in the one-letter code designates the new residue(s) introduced at each position. The numbering is according to [Ala]interleukin 2 [8, 9]. The segments, i.e. helices and helix-connecting loops, are taken from [11]. The second exchange at T125 is not considered

| Mutein    | Affected segment |
|-----------|------------------|
| Wild type |                  |
| S125      | F                |
| A125      | F                |
| T125      | F                |
| Q15       | A                |
| Q15/T125  |                  |
| N20       | AB               |
| N20/T125  |                  |
| Q32       | AB               |
| Q32/T125  |                  |
| Q35       | B                |
| Q35/T125  |                  |
| Q38       | B                |
| Q43       | B                |
| Q43/T125  |                  |
| Q48/T125  | B'               |
| Q49/T125  | B'               |
| Q52       | B'               |
| Q54/T125  | B'               |
| Q60/T125  | B'C              |
| Q61/T125  | B'C              |
| Q62/T125  | B'C              |
| Q76/T125  | C                |
| N84/T125  | D                |
| Q97       | D                |
| Q100/T125 | D                |
| Q110/T125 | E                |
| Q116      | EF               |
| Q120/T125 | F                |
| K19/T125  | AB               |
| K25/T125  | AB               |
| K44/T125  | B                |
| K94/T125  | D                |

Table 2. Yield of interleukin 2 and muteins during purification. The muteins are described in Table 1

| Fraction           | Yield of IL2 and muteins |          |            |
|--------------------|--------------------------|----------|------------|
|                    | K44/T125;<br>Q110/T125   | K94/T125 | all others |
|                    | % total                  |          |            |
| Inclusion bodies   | = 100                    | = 100    | = 100      |
| After CM-Sepharose | < 0.1                    | 27–40    | 1.9–4.6    |
| After HPLC         | < 0.1                    | 22–38    | 0.8–2.4    |

The removal of Cys-125 does not influence renaturation yields to a major extent. The S125, A125 and T125 muteins are recovered during the present protocol in the 0.8–2.5% range observed with most of the other muteins. Some exchanges have been constructed in the Thr-125 background as well as in the Cys-125 background; similar results were obtained.

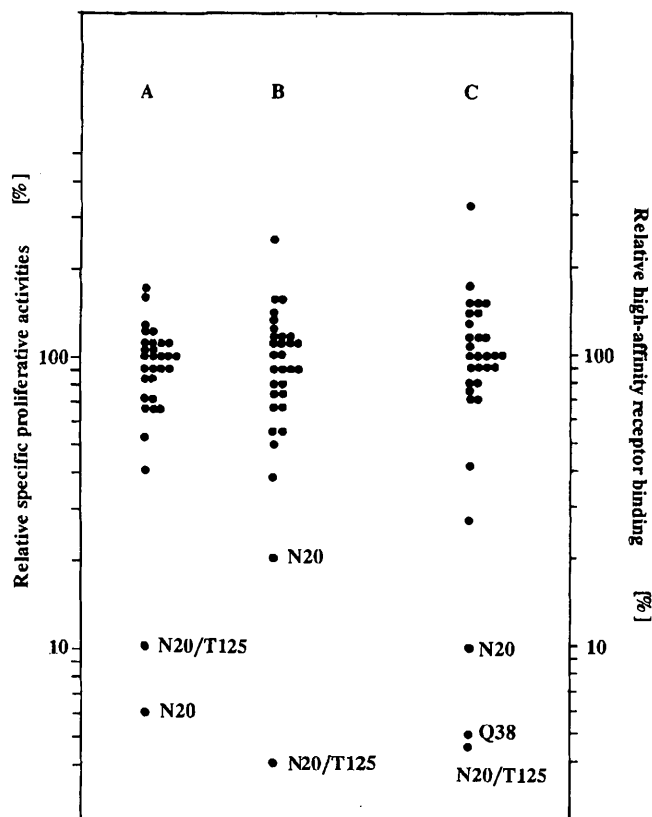


Fig. 2. Proliferative activity and high-affinity receptor binding of re-natured and purified II2 muteins. II2 wild type and the muteins listed in Table 1 were analysed in a proliferation assay with murine CTLL2 cells (A) and human concanavalin-A-stimulated blast cells (B). The mean value of the specific activities of the wild-type-like muteins was taken as 100% ( $3 \times 10^7$  U/mg protein for CTLL2 cells and ConA blasts). (C) The concentration of a mutein causing half-maximal reduction of  $^3\text{H}$ -labeled mutein T125 binding ( $\text{IC}_{50}$ ) was related to the  $\text{IC}_{50}$  of mutein T125. The ratio ( $\times 100$ ) of  $\text{IC}_{50}$  for T125/ $\text{IC}_{50}$  for mutein is plotted

#### Proliferative activity of II2 muteins with murine and human T cells

The re-natured and thoroughly purified muteins were analysed for their biological activity in a proliferation assay with murine CTLL cells and with human concanavalin-A-activated T lymphocytes. The resulting specific activities were related to the mean value of all measurements which was set as 100% (Fig. 2). The specific activity of wild-type II2 varied between  $2-4 \times 10^7$  U/mg protein during these measurements.

Most muteins exhibited specific activities similar to that of the wild type and with a variability among the muteins similar to the variability among multiple measurements of the same mutein. Two notable exceptions occurred, however. The muteins N20 and N20/T125 both showed specific activities significantly lower than that of the other proteins. This applied for the assay with both the murine and human T cells. Since the Thr-125 exchange alone had no effect, the exchange of Asp-20 to Asn was identified independently in the two muteins to cause an about 10–20-fold reduced biological activity.

#### Binding of the II2 muteins to the human high-affinity receptor

The binding of the muteins to the high-affinity receptor present on HUT 102 cells was estimated in competition exper-

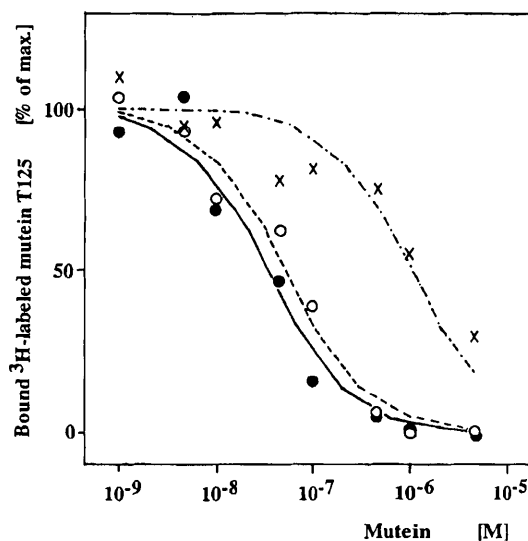


Fig. 3. Competition of II2 and muteins for the low-affinity receptor. HUT 102 cells were incubated with mixtures of  $^3\text{H}$ -labeled mutein T125 (10 nM) and the concentrations of the unlabeled muteins indicated in the abscissa. Bound  $^3\text{H}$ -labeled mutein T125 was determined after competition with unlabeled muteins T125 ( $\bullet$ ), N20 ( $\circ$ ), and Q38 ( $\times$ )

iments employing mass- $^3\text{H}$ -labeled mutein T125. The  $^3\text{H}$ -labeled II2 was present in the binding assay at 250–500 pM. This concentration is between the dissociation constants of the high- and the low-affinity receptor, which were about 50 pM and 20 nM, respectively, in the experiments described. The concentration of a mutein necessary for half-maximal competition of the  $^3\text{H}$ -labeled mutein T125 was determined. The binding potencies, i.e. the ratio of  $\text{IC}_{50}$  for II2 to  $\text{IC}_{50}$  for mutein is compiled for all muteins in Fig. 2C.

The receptor binding was similar to the wild type for most of the muteins. However, muteins N20 and N20/T125 had a 10–20-fold lower potency, in line with results of the proliferation assays.

Surprisingly, the mutein Q38 also had a much reduced potency as confirmed with different preparations. This result is puzzling, since mutein Q38 exhibited completely normal specific activities during the proliferation assays.

For some muteins, i.e. K19/T125, K25/T125 and Q16, slightly reduced or increased potencies in receptor binding were measured. These proteins were not further analysed in the present study.

#### Binding of muteins N20 and Q38 to the low-affinity receptor

The altered binding affinities of muteins N20, N20/T125, and Q38 to the high-affinity receptor leads to several questions. First of all, is it possible to observe similar alterations in the binding to the low-affinity receptor?

The results of a competition of these muteins for the binding of 10 nM  $^3\text{H}$ -labeled muteins T125 to HUT 102 cells are shown in Fig. 3. Both the mutein N20 and N20/T125 competed for the low-affinity receptor at the same concentrations as the wild-type-like mutein T125. In contrast, mutein Q38 had a 10–20-fold lower potency.

#### Stability of muteins in guanidinium chloride

The altered functional properties of the above-mentioned muteins might be caused by conformational features of these

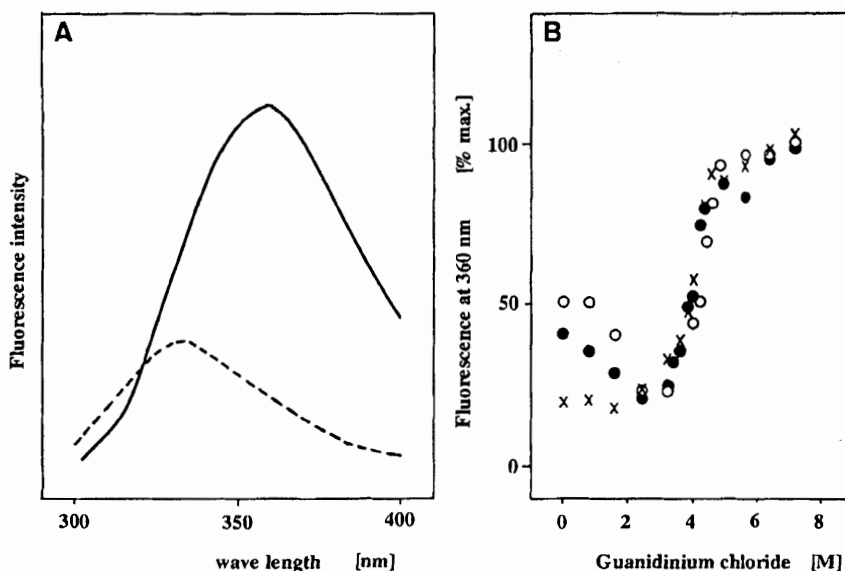


Fig. 4. Guanidinium hydrochloride denaturation curves for selected II2 muteins. Fluorescence emission spectra were recorded for the individual proteins dissolved at the indicated concentrations of guanidinium hydrochloride, as shown in (A) for mutein Q38 dissolved at 1 µg/ml in 25 mM ammonium acetate pH 5 (---) or in the same buffer plus 7.2 M guanidinium chloride (—) (B). The fluorescence intensities at 360 nm were plotted for muteins T125 (●), N20 (○), and Q38 (×)

proteins, despite their normal behaviour during renaturation and isolation. Therefore, their stability in a chaotropic agent was analysed [30].

The single Trp-121 of human II2 exhibits a fluorescence emission spectrum with a maximum at 335 nm. According to fluorescence quenching experiments this Trp is buried [31, 32]. During denaturation in concentrated guanidinium chloride solution the maximum was shifted rapidly, i.e. faster than in 1 s, to 360 nm and the intensity at this wavelength increased about fivefold (Fig. 4A). A reverse rapid shift was observed when the denaturing solution was diluted.

The emission spectra were recorded for muteins N20, Q38 and for the wild-type-like mutein T125 at increasing concentrations of guanidinium chloride. As shown in Fig. 4B, the dose/response curves for the three proteins analysed are very similar at concentrations of guanidinium chloride higher than 2 M. The half-maximal effect is observed at 4 M guanidinium chloride. Thus, the stability of II2 is not affected in the muteins under the conditions applied at least as far the environment of Trp-121 is concerned.

At lower concentrations of guanidinium chloride variable intensities at 360 nm were observed. This appears to reflect a variable degree of denaturation of the different proteins during the lyophilisation from 0.1% trifluoroacetic acid in 60% acetonitrile (see Methods).

## DISCUSSION

The aim of the present study was to identify surface residues in human II2 that provide part of the binding site for the receptor. The experimental approach was to modify amino acid residues by site-directed mutagenesis, to produce the resultant muteins in *E. coli*, and to analyse them for their biological activity. This approach has several intrinsic problems, some of which are difficult to overcome. First of all, structural effects of an amino acid exchange should be separated as far as possible from functional effects. In previous

communications on human and murine II2 muteins, the functional activity of muteins was tested on the level of detergent-solubilised *E. coli* cells or inclusion bodies [16, 18]. While it is quite clear that these protein fractions contain initially a denatured and reduced II2, it is completely uncertain how far the protein renatures during dilution or during the assays. Thus, the activities reported might represent a residual activity of an unfolded or partially refolded protein or the full activity of a small percentage of fully renatured protein. A reduced activity of a mutein may be caused by a defect in refolding or a defect in receptor binding. Actually it would be interesting to know whether the completely unfolded II2 has residual receptor binding activity (see [17]).

In the present study, muteins which survived the applied renaturation and purification procedure were considered to be renatured, i.e. functionally refolded. This operational definition of renaturation appears to be justified since wild-type II2 and most of the muteins are recovered with normal biological activity. A final proof for a renaturation has to await crystallisation and X-ray analysis of a mutein.

Nevertheless, several interesting results were obtained on the present level of sophistication. The lack of renaturation after modification of positions 44 (hydrophobic) and 110 (polar) is most easily explained if the affected side chains are normally buried within the protein and thus contribute to the stabilisation of the tertiary structure. Otherwise, II2 aggregates at neutral pH already at a low concentration ( $K_d$  of 0.6 µM) [31]. Possibly, in the two nonrenaturing muteins the aggregation tendency of a refolding intermediate is enhanced. Modification of juxtaposed positions Lys-43 to Gln and Asp-109 to Asn [33] have no detectable effects. Unfortunately, at this time the three-dimensional structure of human II2 has been resolved to 0.3 nm only [11]. This low resolution does not distinguish whether the substituted residues are located at the surface or the interior of the protein.

The overall yield of renatured and purified II2 generally was low (0.8–2.4%). The finding that the modification of hydrophobic position Leu-94 to Lys enhances the yield more

than tenfold came as a surprise. This effect cannot yet be explained. The solubility of the mutein at neutral pH value appears not to be altered to a major extent.

The proliferation assay yielded specific activities for all muteins as high as the wild type, within the accuracy of the determination, with one exception: the alteration of the acidic position Asp-20 to Asn reduced activity more than tenfold. The same position had been substituted isofunctionally (Asp to Glu) previously without effect on the activity of the denatured protein [16]. The drastic modification of the juxtaposed hydrophobic position Leu-19 to Lys had no major effect. A fine structural deletion analysis with mouse I12 established that a segment corresponding to human I12 position 18–21 cannot be deleted without interfering with the structure or function of the protein [18]. This segment represents the last turn of helix A and the first residue of the long loop connecting helices A and B [11].

A reduced activity of a mutein in the proliferation assay can have different causes, e.g. reduced stability, reduced affinity for the receptor, or an increased turnover/degradation of the mutein-receptor complex in the cell. Actually, the proliferation activity and binding affinity for the high-affinity receptor decreased in parallel for the two N20 muteins analysed. For all other muteins both properties were as in the wild type, with one puzzling exception. Mutein Q38 binds at least tenfold more weakly to the high-affinity receptor than the wild-type I12, despite the fact that it has unchanged proliferation activity. The basic position 38 has been modified before to an acidic one (Arg to Glu) without effect on the proliferative activity of the denatured protein [16]. How can the peculiar behaviour of mutein Q38 be explained? At the moment, we cannot formally exclude a selective instability under the conditions of receptor binding, e.g. cold sensitivity. One might visualise, however, another possibility that the occupancy of the high-affinity receptor is only one of the determinants of the dose/response curve during the proliferation assay.

The binding studies under low-affinity conditions indicate that an alteration of position 20 (Asp to Asn) does not affect binding to the  $\beta$  subunit (Tac antigen), whereas an alteration of position 38 (Arg to Gln) reduces affinity for this protein to a major extent. It will be interesting to see whether the binding to the  $\alpha$  subunit is inversely influenced by these mutations. At the moment it is tempting to speculate that position Arg-38 represents part of the binding site for low-affinity-binding  $\beta$  subunit, whereas position Asp-20 is involved in the binding to the signal-transducing  $\alpha$  subunit. Possibly only the binding to the  $\alpha$  subunit determines the proliferative activity of an I12 mutein.

Interestingly, previous studies with sequence-specific antibodies [14] revealed that antibodies directed against residues 8–27 inhibited binding of I12 to YT cells ( $\alpha$  subunit) only, while antibodies directed against residues 33–54 specifically inhibited binding to MT-1 cells ( $\beta$  subunit).

We thank C. Harmening and U. Wallner for skilful technical assistance, and Drs R. Cantrill, H. Conradt and U. Schwulera for helpful discussions. This work was supported by *Forschungs- und Entwicklungsvertrag GBF 87/01* and by the *Fonds der Chemischen Industrie*.

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