Mechanism of Suramin-Induced Deoligomerization of Tumor Necrosis Factor α

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Received September 7, 1994; Revised Manuscript Received January 17, 1995[⊗]

ABSTRACT: Deoligomerization of human tumor necrosis factor α (TNF), spiked with ^{125}I-labeled form, was studied quantitatively using size-exclusion chromatography and off-line monitoring with a γ-counter. A detailed investigation of the oligomeric state of TNF was carried out as a function of its own concentration (0.3−7500 nM referred to the subunit, M_r 17 000) in the absence or in the presence of various amounts (10, 100, 1000 μM) of suramin, an inhibitor of TNF biological activity in vitro, which promotes TNF deoligomerization. The dependence of trimeric form content on total TNF concentration was modeled with a sequential dissociation process (trimer → dimer → monomer) assuming an identical dissociation constant for each step, K_{d1} = 0.2 nM. This model was used as the simplest for data fitting although, generally, no chromatographic resolution of dimeric species could be obtained. Best fitting of all data could be achieved with a model including a conformational change of TNF trimer into a state more prone to deoligomerization (K_{d2} = 400 nM), which was favored by suramin binding. A kinetic study of TNF dissociation by the same method produced values for the deoligomerization rate of trimer: on the average, $k_{off} \approx 4 \times 10^{-5}$ s^{-1} ($t_{1/2} \approx 5$ h) between 4 and 20 °C with little dependence on suramin concentration; at 37 °C, a sizable increase is observed in the presence of 1 mM suramin ($k_{off} = 2.3 \times 10^{-4}$ s^{-1}, $t_{1/2} = 0.8$ h). Data of suramin inhibition on TNF receptor binding, as obtained after incubation times much shorter than the above half-life of trimer, indicate that suramin binding to TNF trimer is the early mechanism of receptor binding inhibition.

Suramin, a well-known drug for the treatment of trypanosomiasis and onchocerciasis (Hawking, 1978) and currently under evaluation as an anticancer agent (La Rocca, 1990), has been found to inhibit the interaction of human tumor necrosis factor α (TNF)[1] with its cellular receptors (Grazioli et al., 1992) and to promote dissociation of trimeric TNF into inactive subunits (Alzani et al., 1993). To the latter point determinant was the observation that gel filtration chromatography on Superose 12 with 1 mM suramin in the eluting buffer could resolve a trimeric form and another, probably monomeric form. Chemical cross-linking coupled with SDS gel electrophoresis (Lam et al., 1988) proved capable of resolving TNF trimers, dimers, and monomers, but no quantitative use of this technique has been attempted because of the unknown yield of cross-linking reaction for different TNF species and of unpredictable perturbation of oligomerization equilibrium. On the contrary, gel permeation in the presence of suramin produces a predictable perturbation of the oligomerization equilibrium during transit time in the column. After determination of the first-order dissociation rate for the trimer in the presence of 1 mM suramin, this effect was found small and could be easily corrected for in all data. Therefore, gel permeation is used here to investigate the equilibrium and the kinetics of TNF deoligomerization as a function of its own concentration in the absence and in the presence of suramin.

Considerable experimental evidence has accumulated about the instability of the aggregated form of TNF at subnanomolar concentration (Smith & Baglioni, 1987; Petersen et al., 1989; Aderka et al., 1992; Corti et al., 1992), but a quantitative description of the relevant equilibrium is still lacking. Although no precise value is available for the equilibrium dissociation constant, K_d, estimates for the half-life of oligomers vary from 3 to 4 h (Kunitani et al., 1988) up to about 20 h (Corti et al., 1992; Poiesi et al., 1993) according to the experimental method and conditions. After a preliminary evaluation of deoligomerization kinetics, which was needed for a reasonable estimate of equilibrium time, equilibrium data have been collected in a wide range of TNF and suramin concentrations and analyzed with several models. This study has been carried out with refrigerated solutions (4 °C) where formation of high molecular weight TNF aggregates (Aderka et al., 1992) is not interfering with the deoligomerization equilibrium. Finally, suramin inhibition of TNF trimer binding to cellular receptors has been investigated with binding experiments carried out with incubation times much shorter than the half-life of trimers.

MATERIALS AND METHODS

Materials. Materials were obtained from the following sources: recombinant human TNF expressed in yeast and *Escherichia coli* from WOC and Genzyme, respectively; iodinated recombinant human TNF (^{125}I-TNF) from Du Pont−New England Nuclear; suramin (Germanin) from Bayer; acetylavidin-biotinylated horseradish peroxidase com-

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⊗ Abstract published in *Advance ACS Abstracts,* April 15, 1995.
[1] Abbreviations: TNF, human tumor necrosis factor α; p55-s-TNFR, soluble portion of human TNF receptor p55; BSA, bovine serum albumin; cpm, counts per minute; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; HEPES, 4-(2 hydroxyethyl)-1-piperazineethanesulfonic acid.

0006-2960/95/0434-6344$09.00/0

plex from Società Prodotti Antibiotici; *o*-phenylenediamine from Chemicon; bovine serum albumin (BSA) from Miles; bis(2-ethylhexyl) phthalate and dibutyl phthalate (1:1.5) from Fluka; gelatin from Biorad; Tween 20 from Merck, p55-s-TNFR (extracellular portion of the human TNF receptor p55, with M_r 20 000) was expressed in *E. coli* and provided by Dr. M. B. Saccardo, Pharmacia, Nerviano. Recombinant human biotinylated TNF was obtained as described elsewhere (Alzani et al., 1993). The total concentration of TNF is always expressed in terms of subunit with molecular weight 17 000. K562 cells [human erythroleukemia; cf. Lozzio and Lozzio (1975)] were cultured in Dulbecco's modified Eagle's medium (DMEM, Bio-Whittaker) with 10% fetal calf serum (PAA Labor), maintained at 37 °C in the presence of 5% CO_2.

Solid-Phase TNF−Receptor Binding Assay. Plates (96 flat-bottom wells, Falcon-Becton Dickinson 3912) were coated overnight at 4 °C with p55-s-TNFR at a concentration of 200 ng/well in 0.1 M carbonate−bicarbonate buffer, pH 9.6 (100 μL/well). After saturation for 3 h at room temperature in PBS containing 0.25% gelatin (200 μL/well), plates were washed in PBS supplemented with 0.1% Tween 20. Then 10 ng of biotinylated TNF without or with different concentrations of suramin was added to each well in PBS containing 0.1% BSA (100 μL/well). The incubation was performed for 1 h at 20 °C. After several washings, plates were incubated for 1 h at 37 °C with acetylavidin-biotinylated peroxidase complex diluted in PBS supplemented with 0.25% gelatin (100 μL/well). Wells were thoroughly washed, and *o*-phenylenediamine substrate solution (100 μL/well) was added. The reaction was stopped with 4.5 M H_2SO_4 (100 μL/well), and the optical density (OD) was read at 490 nm. Results were expressed as the percent ratio of OD values measured for samples containing TNF and suramin versus those of samples containing TNF alone.

Cell Binding Assay. K562 cells (2×10^5 in 100 μL) were incubated for 1 h at 4 °C in a binding buffer (DMEM supplemented with 3% fetal calf serum and 14 mM HEPES) with ^{125}I-TNF (0.3 nM) in the presence or absence of unlabeled TNF (30 nM) or different concentrations of suramin (200 μL total volume). At 37 °C, incubation (10 min) was carried out in the same binding buffer supplemented with 0.02% sodium azide. At the end of incubations, cell viability was greater than 90% even in the presence of suramin. 150 μL of each cell suspension was then centrifuged through a phthalate−oil mixture. Tips of the centrifuge tubes containing the pellets were cut, and cell-bound radioactivity was determined by a γ-counter. Specifically bound values were calculated as

$$\text{specifically bound TNF (\%)} = 100 \times \frac{\text{cpm}_2 - \text{cpm}_0}{\text{cpm}_1 - \text{cpm}_0} \quad (1)$$

cpm_0 = total counts of reference samples containing 0.3 nM ^{125}I-TNF and 30 nM cold TNF; cpm_1 = total counts of samples containing 0.3 nM ^{125}I-TNF without suramin; cpm_2 = total counts of samples containing 0.3 nM ^{125}I-TNF and suramin.

FPLC Gel Filtration Chromatography. Gel filtration chromatography was performed on a Superose 12 column (Pharmacia Biotech) using an FPLC apparatus (Pharmacia Biotech). The column was equilibrated and eluted in PBS containing 0.05% BSA, 0.02% NaN_3, and 1 mM suramin at a flow rate of 0.5 mL/min. In all described experiments, various concentrations of TNF were spiked with ^{125}I-TNF (0.3 nM), and the mixtures were then incubated for 48 h at 4 °C to equilibrate labeled and unlabeled TNF in PBS containing 0.05% BSA and 0.02% NaN_3. For experiments of TNF dissociation kinetics, spiked and preequilibrated TNF (120 nM) was incubated with 1 mM suramin at 4, 20, and 37 °C and chromatographed after different times. The rate of spontaneous dissociation was similarly investigated at 4, 20, and 37 °C upon diluting thermostated radioiodinated TNF from about 50 nM to 0.3 nM. For equilibrium experiments, various concentrations of TNF were further incubated for 48 h at 4 °C with or without different doses of suramin and then chromatographed. Fractions of 250 μL were collected and tested for radioactivity in a γ-counter. All gel filtration chromatographies were performed at 20 °C.

Resolution of chromatographic peaks in terms of Gaussian or Lorentzian bandshapes was obtained with the curve-fit facility of the program SigmaPlot (Jandel Scientific, Erkrath, Germany) and relative areas were derived analytically from best-fitting values of band amplitude and spread. The mole fraction of TNF subunits in trimeric form (fraction of trimer) was assigned to the relative area of the peak eluting at lower volumes. In the case of samples containing less than 1 mM suramin in the incubation medium, the fraction of trimer was corrected for the extra amount of dissociation introduced by the chromatography (about 20 min at 1 mM suramin) on the basis of the measured rate of dissociation at 20 °C (*vide infra*). This correction never exceeded 5%.

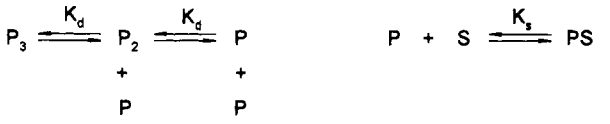
Models for TNF Deoligomerization Data Fitting. Three models have been used for data analysis. In general, the association−dissociation equilibrium of TNF is modeled as a sequential process (trimer → dimer → monomer) described by one parameter (K_d or K_{d1}, dissociation constant of each step). In the simplest model, the suramin effect is accounted for by exclusive binding to the monomeric form (dissociation constant K_s). At an intermediate level of complexity, suramin binding to TNF trimer is allowed (dissociation constant K_{s2}), but binding to dimer and monomer (dissociation constant K_{s1}) is preferred. Finally, according to an allosteric model, TNF is supposed to exist in two conformations: R and T. In the absence of suramin, R is the favored form ($[T_3]/[R_3] = \alpha \ll 1$), but the two trimers have different stability with respect to deoligomerization (K_{d1} and K_{d2} are the dissociation constants of trimers into dimer and monomer, $K_{d2} > K_{d1}$). In agreement with the Monod−Wyman−Changeux formulation of allostery, aggregated states containing subunits in the two conformations (*e.g.*, R_2T) are completely neglected (Monod et al., 1965). Suramin acts by selective binding, and thus stabilizing, each TNF subunit in the T-conformational state (in the trimeric, dimeric, or monomeric form). K_s is the dissociation constant of this suramin−T subunit complex. A concise description of the three models is shown in Scheme 1, which also illustrates the significance of the parameters used in model fitting. The pertinent equation is shortly derived here for the most complex case (allosteric model); in this case, mass balance in terms of total TNF subunit concentration, c_P, reads

$$c_P = 3(R_3 + T_3 + T_3S + T_3S_2 + T_3S_3 + X_3) + 2(R_2 + T_2 + T_2S + T_2S_2) + R + T + TS + D \quad (2)$$

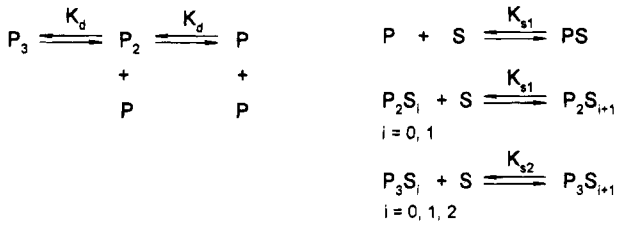
Two species have been introduced beyond these described

Scheme 1

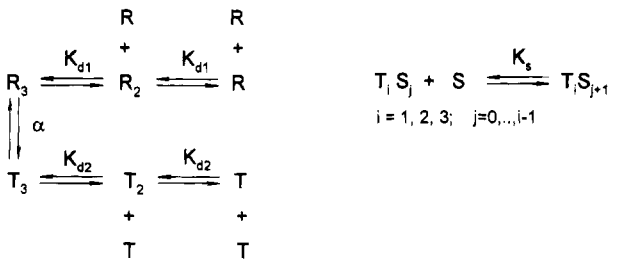
Model 1. Exclusive suramin binding to TNF monomer



Model 2. Preferential suramin binding to TNF monomer and dimer



Model 3. Allosteric mechanism



in Scheme 1: X_3 is a covalently bridged, nondissociable trimeric form; D is a denatured, nonassociating monomeric form. The existence of these species is inferred from the maximum and the minimum value of trimer fraction which can be experimentally obtained. With standard techniques (Cantor & Schimmel, 1980), the concentration of each species in eq 2 can be readily expressed as a function of the free monomer concentration in the R-state, [R], from the equilibria depicted in Scheme 1. After substitution in eq 2, one obtains

$$c_P = \frac{3[1+\alpha(1+[S]/K_s)^3]}{K_{d1}^2}[R]^3 + \frac{2[1+\sqrt[3]{\alpha^2(K_{d1}/K_{d2})}(1+[S]/K_s)^2]}{K_{d1}}[R]^2 + [1+\sqrt[3]{\alpha(K_{d1}^2/K_{d2}^2)}(1+[S]/K_s)][R] + 3[X_3] + [D] \quad (3)$$

For computational convenience, the following adimensional parameters are introduced: x, the mole fraction of TNF as monomer in the R-state, $\sigma = [S]/K_s$, $\beta = K_{d1}/K_{d2}$, $\underline{K}_{d1} = K_{d1}/c_P$, $x_{min} = 3[X_3]/c_P$, and $x_{max} = [D]/c_P$. The two latter quantities are independent of total TNF or suramin concentration and are treated as free parameters.

$$1 = \frac{3[1+\alpha(1+\sigma)^3]}{\underline{K}_{d1}^2}X^3 + \frac{2[1+\sqrt[3]{\alpha^2\beta}(1+\sigma)^2]}{\underline{K}_{d1}}X^2 + [1+\sqrt[3]{\alpha\beta^2}(1+\sigma)]X + x_{min} + x_{max} \quad (4)$$

The resulting cubic equation (eq 4) has one root, $0 < x_0 < 1$, which is evaluated numerically with a procedure included in the software SCIENTIST (Micro-Math, Salt Lake City, UT). The fraction of subunits in the trimeric form is then computed as

$$\frac{3(R_3+T_3+T_3S+T_3S_2+T_3S_3+X_3)}{c_P} = \frac{3[1+\alpha(1+\sigma)^3]x_0^3}{\underline{K}_{d1}^2} + x_{min} \quad (5)$$

Similarly, the following cubic equations are derived for models 1 and 2 ($\sigma_1 = [S]/K_{s1}$ and $\sigma_2 = [S]/K_{s2}$):

$$\text{model 1:} \quad 1 = \frac{3x^3}{\underline{K}_d^2} + \frac{2x^2}{\underline{K}_d} + x(1+\sigma) + x_{min} + x_{max} \quad (6)$$

$$\text{model 2:} \quad 1 = \frac{3(1+\sigma_2)^3x^3}{\underline{K}_d^2} + \frac{2(1+\sigma_1)^2x^2}{\underline{K}_d} + (1+\sigma_1)x + x_{min} + x_{max} \quad (7)$$

and, correspondingly, the fraction of trimer is computed as

$$\text{model 1:} \quad \frac{3(P_3+X_3)}{c_P} = \frac{3x_0^3}{\underline{K}_d^2} + x_{min} \quad (8)$$

$$\text{model 2:} \quad \frac{3(P_3+P_3S+P_3S_2+P_3S_3+X_3)}{c_P} = \frac{3x_0^3(1+\sigma_2)^3}{\underline{K}_d^2} + x_{min} \quad (9)$$

For selected values of the parameters, calculations are repeated at each concentration of TNF and suramin. Best values of the parameters are finally obtained by the nonlinear least-squares optimization procedure of software SCIENTIST.

RESULTS

Determination of Trimer Fraction. Examples of resolved chromatograms are shown in Figure 1. Data refer to the analysis of deoligomerization kinetics induced by incubating TNF (120 nM) with 1 mM suramin at 4 °C and illustrate resolution with different combinations of peak shapes. The peak at lower elution volume corresponds to the trimeric form, and the second peak contains monomeric and, possibly, dimeric form (Alzani et al., 1993). Resolution into three peaks could be obtained with another gel permeation column (TSK-SW2000XL). This column, however, had been used extensively for the analysis of various heparin preparations (Gigli et al., 1992), and results obtained with a new column, even after priming with beef lung heparin, were inconclusive. As it is clear from Figure 1 (and substantiated by the residuals), a Gaussian bandshape for the first peak and a Lorentzian bandshape for the second peak generally perform better at data fitting than two Gaussian bandshapes. With the mixed-bandshape resolution, column recovery after incubation of TNF solutions at 4 °C, as estimated from the sum of the two peak areas versus the known load of radiolabeled TNF, is nearly quantitative (96.3 ± 9.8% as average of 93 experiments). This result agrees with the very limited amount (<5%) of highly aggregated material eluting

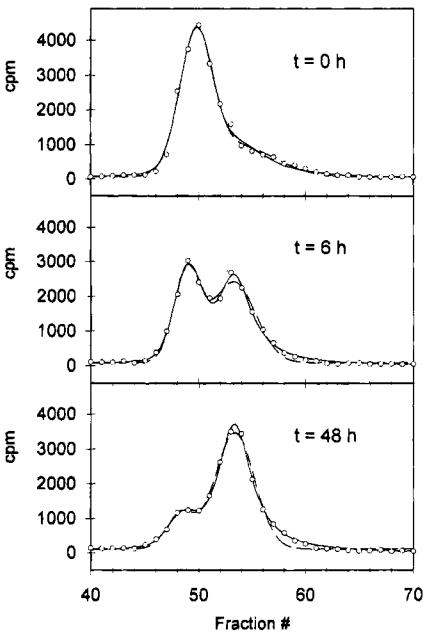


FIGURE 1: Resolution of chromatographic peaks in the presence of various amounts of trimeric and monomeric TNF as obtained during analysis of the deoligomerization kinetics. TNF spiked with 0.3 nM ^{125}I-TNF (total concentration of TNF subunit = 120 nM) was incubated at 4 °C with 1 mM suramin for various times (0, 6, 48 h) and chromatographed on a Superose 12 column. Fractions of 250 μL were collected and tested for radioactivity. Experimental results (empty circles) are expressed as cpm (counts per minute). The solid lines are a result of resolution with a sum of a Gaussian and a Lorentzian peak, whereas resolution with two Gaussian peaks is illustrated by the dashed lines.

at much shorter volumes in these conditions. Incubation at higher temperature (37 °C) generally produced an increase of this extra peak and a consequent poorer recovery. Several equilibrium experiments were repeated with recombinant TNF expressed both in *E. coli* and in yeast, and no appreciable difference of the trimer content was observed within our experimental error, estimated at around 10% of the value. At low total TNF concentration, even in the presence of high suramin concentration, the fraction of subunits in the trimeric state never decreased below 5−7%. This result is attributed to the presence of a small amount of undissociable trimer in the radioiodinated TNF preparation. Consistently, SDS−PAGE experiments in nonreducing conditions (data not shown) reveal, beside the spot corresponding to the monomeric subunit, a secondary one (approximately 8% of the total by photometric scan of autoradiograms) at higher M_r; the intensity of this spot considerably decreases after sample reduction with β-mercaptoethanol.

Kinetics of TNF Deoligomerization. Correct planning of the equilibrium experiments (at 4 °C) required evaluation of the kinetic dissociation constant (k_{off} at 4 °C) of TNF trimer. Equilibrations both of the radioiodinated subunits with the bulk TNF and then of oligomer−monomer with suramin are dependent on k_{off}. Moreover, the value of k_{off} at 20 °C was necessary for evaluation of the correction factor accounting for trimer dissociation during its transit through the gel permeation column. Kinetic experiments were then extended to 37 °C in order to provide a link with biological results. As an example, the deoligomerization kinetics of TNF (120 nM) incubated at 4 °C in the presence of 1 mM suramin are reported in Figure 2. Both in the presence and

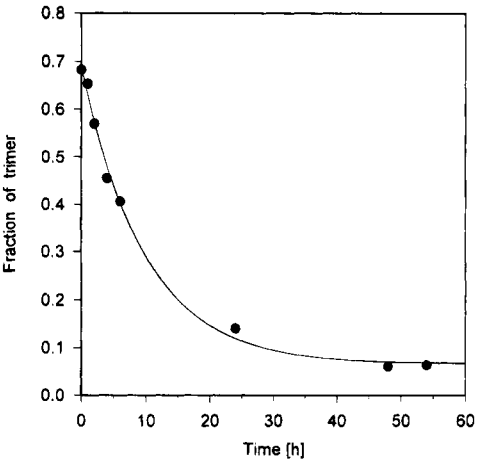


FIGURE 2: Kinetics of TNF deoligomerization induced by 1 mM suramin at 4 °C. The total concentration of TNF subunits was 120 nM, spiked with 0.3 nM ^{125}I-TNF. Data of this experiment are analyzed with one exponential ($k_{off} = 2.85 \times 10^{-5}$ s^{-1}). The value reported in the text is the average of three determinations.

Table 1: Best-Fitting Parameters of Suramin−TNF Interaction Models[a]

parameter	model 1	model 2	model 3
K_d or K_{d1} (nM)	0.7 (0.3)	0.9 (0.2)	0.2 (0.1)
K_{d2} (nM)			400 (70)
K_s or K_{s1} (μM)	4.3 (1.2)	27 (6)	3.7 (1.7)
K_{s2} (μM)		110 (30)	
α			4 (6) × 10^{-6}
x_{min}	0.10 (0.02)	0.05 (0.03)	0.09 (0.01)
x_{max}	0.09 (0.03)	0.02 (0.03)	0.09 (0.03)
$\Sigma(y_{fit} - y_{dat})^2$	0.089	0.071	0.031

[a] Standard deviations of parameter estimates are in parentheses.

Table 2: Kinetic Dissociation Constants of TNF Trimer (k_{off}, 10^{-5} s^{-1})[a,b]

suramin concn (mM)	T (°C)		
	4	20	37
0	4.4 ± 1.5	4.3 ± 0.7	6.1 ± 0.6
1	3.1 ± 0.6	4.4 ± 1.1	23 ± 6

[a] Data at 4 and 37 °C are averages of two or three independent experiments with corresponding standard deviations; data at 20 °C are results of a single kinetic experiment with standard deviation of parameter estimates. [b] Trimer half-life, $t_{1/2}$, is obtained from k_{off} as follows: $t_{1/2}$ (h) = 19.3/k_{off} (10^{-5} s^{-1}).

in the absence of suramin, data (fraction of trimeric form as a function of incubation time) are very well interpreted by a first-order process, and corresponding values of k_{off} are collected in Table 2 for three different temperatures. In the absence of suramin, k_{off} depends modestly on temperature, and trimer half-life ($t_{1/2}$) varies between 4.5 h at 4 °C and 3.2 h at 37 °C. On the contrary, a sizable increase of dissociation rate with temperature is observed in the presence of 1 mM suramin ($t_{1/2}$ varies from 6.2 h at 4 °C to 0.8 h at 37 °C). Correspondingly, at 4 and 20 °C, the dependence of k_{off} on suramin concentration is blurred within experimental errors, whereas suramin clearly accelerates deoligomerization at 37 °C. This latter result is compatible with a mechanism where the slowest step of the whole deoligomerization process follows suramin binding (Bernasconi, 1976). For instance, dissociation of TNF trimer into dimer and monomer according to models 2 or 3 (see Scheme 1) could be such a step. The opposite dependence (decrease

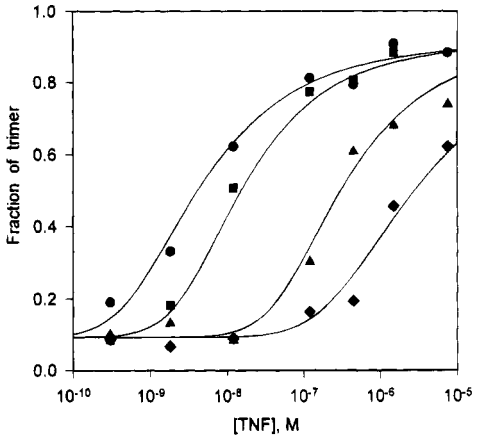


FIGURE 3: Dependence of the TNF trimeric form on the total concentration of subunits in the presence of various amounts of added suramin (circles, 0 mM; squares, 0.01 mM; triangles, 0.1 mM; diamonds, 1 mM). Most data are averages of two chromatographic analyses with TNF of different sources. The difference of the two determinations never exceeded 10%. The curves are a result of best fit with the allosteric model discussed in the text, with parameter values as listed in Table 1.

of k_{off} with increasing suramin concentration) would be expected for a combination of the allosteric model (model 3) and protein isomerization as the slowest step. Trimer dissociation according to model 1, where suramin binds only TNF monomer, would result in a dissociation rate nearly independent of suramin concentration and is not compatible with results obtained at 37 °C. The reason why the suramin effect is only evident at 37 °C probably depends on experimental factors (at lower temperature, errors overcome the dependence of k_{off} on suramin concentration) and on different activation energies for dissociation of the most populated species in each case (according to model 2 or 3: P_3 or R_3 in the absence of suramin, P_3S_3 or T_3S_3 in the presence of suramin).

Formation of high molecular weight aggregates (multimers) becomes important at 37 °C, in agreement with former observations (Aderka et al., 1992). In one experiment, after 48 h incubation in the presence of 1 mM suramin, nearly 40% of all radioactivity was found in an extra peak at short retention volumes. Multimer formation at the expense of a denatured monomeric species seems likely. Growth of the multimer peak is, in fact, much slower than decrease of trimer peak area, and, as a consequence, our estimate of k_{off} at 37 °C should be unaffected. Nevertheless, poor reproducibility of this aggregation phenomenon in repeated experiments strongly advised collection of equilibrium data under refrigerated conditions (4 °C).

Model Fitting to Equilibrium Data. The fraction of TNF trimer at equilibrium, as determined by gel filtration, is plotted in Figure 3 as a function of TNF concentration at several values of suramin concentration (0, 0.01, 0.1, 1 mM). The curves are a result of best-fitting with the allosteric model and the values of the parameters as listed in Table 1. The result of fitting with the other two models is not shown, as the corresponding curves give a poorer representation of data (see total quadratic deviation in Table 1). Not unexpectedly, standard deviations of the parameter estimates are comparatively higher for models with more parameters (e.g., model 3), and in one case, the conformational equilibrium constant α, the standard deviation exceeds the best-fitting

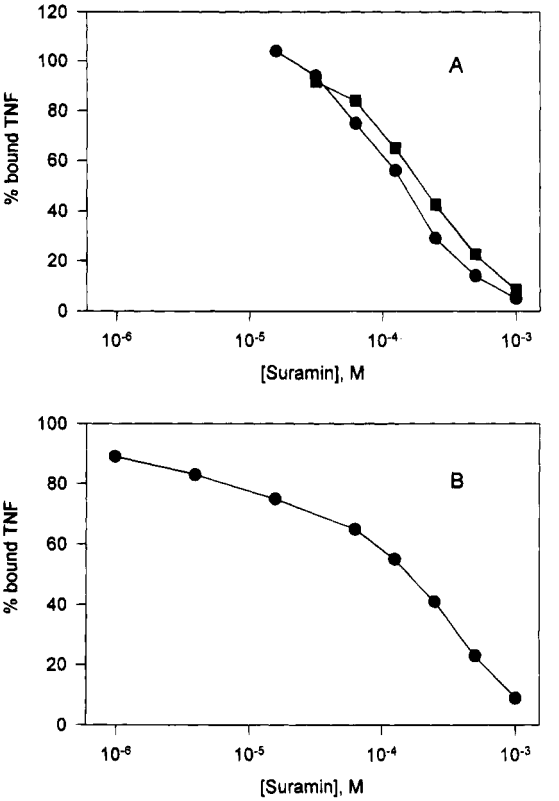


FIGURE 4: Suramin-promoted inhibition of TNF receptor binding: (A) Specific binding of ^{125}I-TNF (0.3 nM final concentration) to 2 × 10^5 K562 cells in the presence of various concentrations of suramin after 1 h incubation at 4 °C (filled circles) and 10 min incubation at 37 °C (filled squares). Results are expressed as percent relative to values obtained in the absence of suramin. (B) Binding of biotinylated TNF (6 nM final concentration) to immobilized p55-sTNFR in the presence of various amounts of added suramin. Biotinylated-TNF and suramin were added to the wells of a 96-well plate coated with 200 ng of p55-sTNFR and incubated for 1 h at 20 °C. In these two plots, lines have been drawn to facilitate recognition of data trends and are not a result of simulation.

value of this parameter. Possibly, a better feeling of the significance of each equilibrium constant can be obtained from a comparison among results obtained with different models. Interestingly, data analysis reveals the presence of a fraction of nondissociable TNF (about 10%, cf. lower plateau in Figure 3) and of a residual nonassociating form (10% or less, cf. upper plateau of Figure 3). The former is assigned to a small amount of cross-linked species (probably through disulfide bridges), in agreement with SDS−PAGE analysis in nonreducing conditions; the latter could be due either to a denatured monomeric form or to an artifact of peak deconvolution with symmetric bandshapes. Peak tailing of the trimeric species in the gel filtration chromatograms would result in an apparent excess of dissociated species.

TNF Binding to Cellular and Recombinant Receptors. The inhibitory effect of suramin in a binding assay between ^{125}I-TNF and its receptors expressed on K562 cells was investigated with very short incubation times (1 h at 4 °C, 10 min at 37 °C), a condition which greatly reduces the importance of deoligomerization, as only about 10% of the trimer dissociates in this time according to our k_{off} estimate. Nevertheless, experimental data (Figure 4A) show dose-dependent inhibition of TNF binding with nearly complete inhibition obtained in the presence of 1 mM suramin. A similar result is obtained in a solid-phase binding assay using recombinant receptor p55-sTNFR and biotinylated TNF

(Figure 4B) after short (1 h at 20 °C) incubation time. Interestingly, the apparent IC_{50}'s of these three experiments are very similar (100−200 μM) and correspond closely to that obtained after much longer (4 h) incubation time (Grazioli et al., 1992). In combination with the kinetic experiments, these results strongly support the hypothesis that suramin is able to rapidly bind TNF trimers and inhibit receptor binding.

DISCUSSION

Though this work was aimed at elucidating the mechanism of interaction between suramin and TNF, the use of this drug in the chromatographic elution buffer proved instrumental for the determination of equilibrium and kinetic parameters of TNF deoligomerization, which are of wider application and significance. In particular, suramin helps by reducing the interaction of nontrimeric TNF to the stationary phase with considerable improvement of the elution pattern (Alzani et al., 1993), and, moreover, suramin prevents reassociation of TNF, a comparatively fast process ($t_{1/2}$ < 10 min at c_P > 10 nM, as estimated from our k_{off} and K_d values) with respect to the time of chromatographic separation (~20 min). However, the derivation of physicochemical parameters from chromatographic data requires some assumption: modeling the dissociation of TNF trimer according to a sequential process (trimer → dimer → monomer) is required by the need of limiting the number of free parameters in data fitting. Nevertheless, a characteristic feature of this model, the existence of a sizable concentration of dimeric species at equilibrium, is supported by some experimental evidence. The chromatographic peak corresponding to the low molecular weight form of TNF is broad and is better described with a Lorentzian rather than a Gaussian bandshape, a feature suggesting a composite (dimer + monomer) nature of this peak; in addition, a clear though nonreproducible resolution of the whole chromatogram into three peaks has been mentioned.

The best estimate of the equilibrium dissociation constant of TNF trimer, K_d = 0.2 nM according to the allosteric model, somewhat increases (up to 0.9 nM) if other models are used for the interpretation of the suramin effect. In spite of this broad model-dependent indetermination, these values are very well consistent with the current picture of TNF as a transiently stable trimeric state at physiological concentrations (Aderka et al., 1992; Corti et al., 1992). The rate of trimer dissociation ($k_{off} \approx 4 \times 10^{-5}$ s^{-1} or $t_{1/2} \approx 5$ h, between 4 and 20 °C) corresponds very well with that estimated using hydrophobic interaction chromatography (Kunitani et al., 1988) but is significantly higher than values obtained with other techniques (Poiesi et al., 1993): $k_{off} = 0.92 \times 10^{-5}$ s^{-1}, biospecific interaction analysis; and $k_{off} = 1.1 \times 10^{-5}$ s^{-1}, immunochemical methods. The reason for this discrepancy is unknown and can be discussed only speculatively. Different methods discriminate the aggregation states of TNF in a different way: chromatography distinguishes trimer from monomer−dimer, whereas the other above methods distinguish aggregated forms (trimers, dimers, and multimers) from monomer. Accordingly, the half-life of TNF trimer, as measured by gel filtration chromatography, could possibly differ from the apparent average half-life of all aggregated forms.

In addition to the characterization of the equilibrium and kinetics of dissociation of TNF trimer, our data quantitate the interaction of suramin with the protein subunits in terms of a dissociation constant in the micromolar range. In analogy with results of studies on the interaction between suramin and some growth factors (Middaugh et al., 1992), the mechanism by which suramin inhibits TNF−receptor binding involves specific binding to the protein in its functional, trimeric state. The consequences of suramin binding differ in the two cases, as TNF dissociates into subunits whereas growth factors generally aggregate after binding, but both these effects seem mediated by a conformational change of the protein. Data fitting of our experimental results supports an allosteric model (model 3 of Scheme 1) involving conversion of TNF trimer into a state more prone to deoligomerization (K_{d2} = 400 nM) upon suramin binding. Because this new conformational state is energetically unfavored, effective suramin−TNF binding requires concentrations much higher than K_s (3.7 μM) and comparable to the IC_{50} detected in biological experiments (>100 μM). Quantitative correlation with biological data would require highly sophisticated models and is out of the scope of the present work. Nevertheless, however complex the model, the description of the equilibrium and kinetics of TNF dissociation in bulk solution should be consistent with the parameters listed in Tables 1 and 2. Some indetermination of the dissociation model remains, and definitive verification of the allosteric model has to wait for structural studies or for direct binding measurements. In fact, model 2 considering preferential (but nonexclusive) suramin binding to monomer and dimer is not incompatible with our data. Model 1 (selective suramin binding to TNF monomer) is definitely ruled out by data on receptor binding inhibition at short incubation times, preceding the deoligomerization. Therefore, inhibition of TNF bioactivity by suramin (Alzani et al., 1993) appears a complex phenomenon that involves direct inhibition of TNF trimer binding to the p55 receptor as well as dissociation of TNF into subunits.

ACKNOWLEDGMENT

We thank Dr. M. B. Saccardo for the generous gift of p55-s-TNFR. Photometric readings of autoradiograms by Mr. G. Razzano are gratefully acknowledged.

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BI942126C