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Analysis of ligand binding to proteins using molecular dynamics simulations

M.R. Housaindokht [a,b,*], M.R. Bozorgmehr [a,b], M. Bahrololoom [a]

[a] Biophysical Chemistry Laboratory, Department of Chemistry, Faculty of Science, Ferdowsi University of Mashhad, P.O. Box 91775-1436, Mashhad, Iran
[b] Research and Technology Center of Biomolecules, Faculty of Science, Ferdowsi University of Mashhad, Mashhad, Iran

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ABSTRACT

This work aims to explore theoretically the molecular mechanisms of ligand binding to proteins through the use of molecular dynamics simulations. The binding of sodium dodecyl sulfate (SDS) to cobra cardio toxin A3 (CTX A3) and thiourea (TOU) to lysozyme have been chosen as the two model systems. Data acquisitions were made by Gromacs software. To begin with, the collisions of ligand molecules with every residue of CTX A3 and lysozyme were evaluated. With this information in hand, the average numbers of collisions with each residue was defined and then assessed. Next, a measure of the affinity of a residue, P_i, referred to as conformational factor, toward a ligand molecule was established. Based on the results provided, all site-making residues for CTX A3 and lysozyme were identified. The results are in good agreement with the experimental data. Finally, based on this method, all site-making residues of bovine carbonic anhydrase (BCA) toward the SDS ligand were predicted.

1. Introduction

The interactions between proteins and other molecules are critical to many biological systems and processes. It has become increasingly clear that signal transduction, metabolic regulation, enzyme cooperativity, physiological response and other processes such as carrying oxygen by hemoglobin are all dependent upon non-covalent binding (Straatsma and McCammon, 1992). Therefore, great stride has been made and a range of techniques developed for studying such interactions.

The equilibrium dialysis, diffusion process, titrometry, calorimetry and various spectroscopic methods are among many techniques used for investigating the binding of a ligand to proteins. In equilibrium dialysis, a bag with a semipermeable wall and containing a protein is placed in the buffer solution. Equilibrium occurs across the membrane such that the free ligand concentration becomes the same both inside and outside the bag, although some of the ligand is bound to the protein; the fraction of ligand bound to the protein, then, can be calculated by making use of the total ligand concentration. This method has been used for studying a number of ligand-binding interactions (Housaindokht et al., 2005a, b; Housaindokht and Moosavi-Movahedi, 1994).

The method based on the diffusion process is, compared with the equilibrium dialysis, both simple and much less time consuming. Besides, it is more realistic, i.e. closer to *in vivo* condition. Therefore, it is quite suitable in the case of unstable proteins (Housaindokht et al., 2002).

Binding of ligand to proteins frequently causes change to their three-dimensional structures, leading to a change of absorption. Accordingly, spectroscopic techniques are valuable tools in studying conformational changes caused by ligand binding. Under these circumstances, however, it is not generally possible to assess the binding constant (Saboury et al., 2002).

Calorimetric methods are used to work out the thermodynamic parameters of binding processes (Housaindokht et al., 1993, 2005a, b). For instance, free-energy changes associated with binding of *n*-alkyl-sulfonates to insulin at various pH values and enthalpy changes of the interaction between glucose-oxidase and a number of *n*-alkyl tri-methyl ammonium bromides homologous at 25 °C were determined by these techniques (Moosavi-Movahedi and Housaindokht, 1990; Jones and Brass, 1991). Titrometric methods, on the other hand, can be used to study proton binding to proteins.

In spite of having many applications, experimental techniques have certain limitations. For example, equilibrium dialysis involves a great deal of time to reach the equilibrium state (ca 90 h). Moreover, ligands may bind to the membrane and this will affect the accuracy of analysis. Spectroscopic techniques are also limited to those situations where the ligand binds to chromophores or to the sites that have an effect on the chromophores. For instance, a fluorescence spectrum is only

* Corresponding author at: Biophysical Chemistry Laboratory, Department of Chemistry, Faculty of Science, Ferdowsi University of Mashhad, P.O. Box: 91775-1436, Mashhad, Iran. Tel.: +98 511 8797022; fax: +98 511 8796416.
E-mail address: mhousaindokht@science1.um.ac.ir (M.R. Housaindokht).

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suitable to determine the binding sites that involve Trp, Tyr and Phe residues. Furthermore, there would be no easily accessible way to assess the contribution made by structural changes, which in most cases is associated with ligand-binding processes, to the overall experimental thermodynamics parameters. In addition, the common approaches in studying the ligand binding to macromolecules are unable to specify the binding sites (Scatchard, 1949; Hill, 1910; Wyman, 1965; Huang and Schroeder, 2006; Beutler and van Gunsteren, 1994; Gilson et al., 1997). For example, to study how new drugs bind to human serum albumin, competition experiments only enable us to determine whether or not the new drug binds to the known sites such as the warfarin binding site (Dufour and Dangles, 2005).

Using computational approaches to investigate and analyze the binding interactions of proteins with ligands can provide useful insights for drug design (Chou, 2004; Chou et al., 2003, 2006; Du et al., 2004, 2005a, b, 2007; Gao et al., 2007; Guo et al., 2007; Li et al., 2007a, b; Lubec et al., 2005; Sirois et al., 2004; Wang et al., 2007a, b, c, d, 2008; Wei et al., 2005, 2006a, b, 2007; Ye et al., 2007; Zhang et al., 2006; Zheng et al., 2007). Among the computational methods developed to predict and analyze protein–ligand binding sites are POCKET (Levitt and Banaszak, 1992), LIGSITE (Hendlich et al., 1997), SURFNET (Laskowski, 1995), CAST (Liang et al., 1998) and LIGSITE^{csc} (Huang and Schroeder, 2006). LIGSITE^{csc} is an extension of LIGSITE. Instead of defining protein–solvent–protein events on the basis of atom coordinates, it uses the Connolly surface and defines surface–solvent–surface events. In the Connolly algorithm, a hypothetical probe sphere (usual radius 1.4 Å) rolls over the protein.

Molecular dynamics simulations, generally, enable investigations of dynamics and conformational changes of biological macromolecules to be conducted and can yield information that would not be available through any other means (Lemaitre et al., 2004; Fraternali et al., 2003; Monticelli et al., 2004; Dairou et al., 2006; Shirts et al., 2003).

In the study of ligand-binding simulations, the free energy of binding is a measure of the complex stability and generally is used in the study of all binding processes (Stewart and McCammon, 2006). Various approaches have been developed to estimate the relative free energy of binding. Among these, free-energy perturbation (FEP) and thermodynamics integration (TI) techniques have been applied in order to predict the accurate free energy of binding a ligand to macromolecules. Among the other methods, one can point out to linear interaction energy (LIE) (Almlöf et al., 1994), molecular mechanics/Poisson Boltzmann surface area (MM/PBSA) (Gilson et al., 1997), chemical Monte Carlo/molecular dynamics (CMC/MD) (Eriksson et al., 1999), four dimensional-potential of mean-force (4D-PMF) (Beutler and van Gunsteren, 1994) and one-window free-energy grid (OWFEG) (Pearlman, 1999). Each one of these methods has been employed to deal with a single kind of ligand binding to macromolecules (Kollman, 1993; Gilson et al., 1997; Eriksson et al., 1999; Randmer and Kollman, 1998).These techniques have their own limitations too. For instance, in spite of being more accurate, TI and FEP methods have major restrictions in that they only allow us to compare binding of two different ligands to one site or binding of one ligand to different sites. Besides, running the calculations is quite more costly (Lindahl et al., 1995).

In this work, a new technique is established to assess the affinity of a residue toward a given ligand in terms of the number of collisions between them, which is obtained by means of dynamics simulations. The interactions of SDS with CTXA3 and thiourea (TOU) with lysozyme have been chosen as the model systems. Based on the presented method all site-making residues of bovine carbonic anhydrase (BCA) toward the SDS ligand were most likely predicted.

1.1. Simulation details

All calculations were carried out using gromacs3.3.1 package (Lindahl et al., 1995) and gromos96 force field (van Gunsteren et al., 1996). The components of simulation boxes for the given systems are listed in Table 1. It should be noted that the number of water molecules, ligands and all other components related to each simulation box have been obtained on the basis of the experimental values reported in references Forouhar et al. (2003), Salem et al. (2006) and Katherine et al. (2005) for CTXA3, lysozyme and BCA, respectively.

A steepest-descent algorithm was performed to minimize the energy of each system and to relax the water molecules.

Molecular dynamics simulation for each system was carried out in two stages. In the first stage, position-restrain simulation was conducted, in which the atoms of the protein molecule were held fixed whereas the water molecules were free to move around so that they would reach the equilibrium state. In the second stage, each system was simulated with a time step of 0.2 fs. LINCS algorithm (Berendsen et al., 1981) was employed to fix the chemical bonds between the atoms of the protein and SETTLE algorithm (Darden et al., 1993) in the case of water molecules. The atoms in the system were given initial velocities according to Maxwell–Boltzmann distribution at 300 °C. To maintain a constant temperature and pressure for various components during simulations, the Brendsen coupling algorithm was used (Hess et al., 1997; Berendsen et al., 1984) for each component of the system with relaxation times of 0.1 and 0.5 ps, respectively. As the systems involve many positive and negative charges, PME algorithm was applied to estimate the electrostatic interactions. In this algorithm every atom interacts with all atoms in the simulation box and all of their images in an infinite number of identical copies surrounding the main box; so satisfactory results are produced from the electrostatic interactions (Danciulescu et al., 2004).

2. Results and discussion

MD simulations make it possible to study the behavior of a protein and a ligand over a specific time span. Due to the motions of the ligand and protein molecule, the ligand could collide with different parts of the protein structure. It can also be expected that the ligand will collide more frequently with binding sites on the protein structure. With this in mind, the following points have

Table 1
Overview of studied systems and simulation detail

Protein	PDB entry	dimension of box	No. of water molecules	Ligand	No. of ligands	Span time of simulation (ns)
CtxA3	1H0J	$10 \times 10 \times 10$	30000	SDS	14	2
Lysozyme	2F4A	$4.6 \times 4.6 \times 4.6$	2602	TOU	100	2
BCA	1V9E	$7 \times 7 \times 7$	10000	SDS	32	5

been considered to evaluate the affinity of a specific residue of the protein molecule toward a specific ligand.

(i) The protein molecule along with a certain number of ligand molecules are assigned to the simulation box (Table 1). The initial positions of the ligand molecules are set up to be random.
The number of collisions of the ligand molecules with every residue of the protein molecule are counted right through the molecular paths resulted by the simulation. When an atom of the ligand molecules is able to reach a distance of 0.4 nm or less from any atom of the protein residue, it is adopted as a collision between the ligand and the residue (Miamoto and Kollman, 1992; Psachoulia et al., 2006; Bond et al., 2004).

(ii) The average number of collisions with each residue over the whole time of simulation is defined as $\langle n\rangle = \Sigma_{i=1}^{N} n_i/N$, where n_i indicates the number of collisions with the ith residue and N the number of residues of the protein molecule. $\langle n\rangle$ can be also identified with any n_i value corresponding to a hypothetical condition in which every residue would have the same affinity toward the ligand. Next, a parameter, referred to as conformational factor, is defined by $P_i = n_i/\langle n\rangle$. Now, in order to have an effective strategy for drawing conclusions from the results concerning the affinity, the following criteria have been adopted:

A residue can be counted as being indifferent toward the ligand when its P_i value turns out to be unity. In other words, when the number of collisions amounts to the average value.

The residue i with $P_i > 1$ is considered to have affinity toward the ligand, while with $P_{i'} < 1$ it has no affinity.

Considering the above points, the following results have been obtained for the proteins studied.

2.1. CTXA3

In the present work, the P_i values of all the residues of CTXA3 structure were calculated (Table 2). Besides, the residues having $P_i > 1$ are presented in Table 3. From the data shown in Tables 2 and 3 the following conclusions can be drawn: most of the residues with $P_i > 1$ are in the loop regions or adjacent to them (Fig. 1). The residues with the hydrophobic nature are also have the $P_i > 1$. This is in accord with the experimental reports (Forouhar et al., 2003) and with the fact that SDS is an amphipathic molecule. The results also reveal that the lysine residue 31 of the third monomer has the largest value of P_i. In other words, this residue would have the greatest affinity towards

Table 2
Calculated P_i values for the CTXA3 residues

Res#	P_i	Res#	P_i	Res#	P_i	Res#	P_i
1	0	51	0.03128	101	0	151	26.9559
2	0	52	0	102	0	152	0
3	0	53	0	103	0	153	0
4	0	54	0	104	25.5102	154	4.13383
5	0.00434	55	0	105	0	155	0
6	2.06778	56	0	106	0.12424	156	18.2251
7	1.7246	57	0	107	0	157	1.67247
8	0.62033	58	0	108	0.79236	158	1.62816
9	0.39705	59	0	109	0	159	0
10	2.36839	60	0	110	0	160	0
11	0	61	0.00956	111	0	161	0
12	0	62	1.8749	112	0	162	0
13	0	63	0	113	0	163	0
14	0	64	0.76021	114	0	164	3.86362
15	0	65	0	115	0	165	0
16	0	66	0	116	0	166	0.01651
17	0	67	0	117	0	167	0.00956
18	0.00434	68	1.00261	118	1.10948	168	0
19	0	69	1.0391	119	0	169	0
20	0	70	0.03128	120	0.03041	170	0
21	0	71	0.36316	121	0	171	0
22	0.18245	72	0	122	0.00087	172	0
23	0	73	0.00348	123	0	173	0
24	0	74	0	124	0.00087	174	0
25	0	75	0	125	2.89316	175	0
26	0	76	0.00348	126	3.61688	176	0
27	0	77	0	127	0	177	0
28	0	78	0	128	0	178	0.11729
29	0	79	0	129	2.00436	179	0
30	0	80	0	130	4.97223	180	0
31	0.08601	81	0	131	6.93836		
32	0.77933	82	0	132	17.1895		
33	0.18071	83	0	133	0.28932		
34	0.00695	84	0	134	0		
35	6.2798	85	0	135	0		
36	0.67941	86	0.01303	136	0		
37	0	87	0.04778	137	0		
38	0	88	0.21634	138	0		
39	0	89	0	139	0		
40	0.00087	90	0	140	0		
41	0	91	13.7933	141	0		
42	0	92	0.02954	142	22.3798		
43	0	93	0	143	0		
44	0	94	0	144	0		
45	0	95	0	145	0		
46	0	96	0.00956	146	0.55604		
47	0	97	0	147	0		
48	0	98	0	148	0.12772		
49	0	99	0	149	0.21634		
50	0	100	0	150	0		

Table 3
The residues having P_i greater than unit

CTXA3	Lysozyme		BCA		
LYS151	TRP28	ASN19	LYS8	ASN23	THR172
LYS104	ASP101	TYR20	LYS250	ASN61	LEU240
TYR142	ASN44	GLN121	LYS170	PHE174	ASP173
ARG156	GLU35	ASN103	LYS157	TRP4	SER28
LYS132	TRP108	TRP111	TRP15	SER171	LEU196
LYS91	TRP62	ASN74	TYR69	SER218	SER164
TYR131	LEU129	SER72	PHE19	ARG225	Pro179
LYS35	TYR23	SER81	PHE177	ARG252	VAL254
PHE130	ASN77	TRP123	SER55	ASP101	THR54
VAL154	ASP87	ASN106	ASP100	LEU238	ASP160
LYS164	ASN65	TYR53	ARG244	ASN230	GLY233
LEU126	GLU7	ASP66	SER181	ALA36	
LYS125	ASN46	LEU84	HIS16	SER98	
PHE10	TRP63	LEU56	ILE21	LEU201	
LEU6	ASN37	LEU17	ASP178	VAL254	
LEU129	ASP52	ARG128	LYS35	ALA231	
LYS62	GLN57	PHE3	ASN251	SER99	
VAL7	ASP18	ARG112	ALA22	GLU234	
GLY157	PHE34	SER85	SER1	SER195	
CYS158	GLN41	ARG45	PRO20	ALA161	
ARG118	ASN59	SER86	GLY169	PHE92	
LEU69	ASP119	THR118	GLU232	GLN91	
PRO68	ASN113		ASN152		

Chain A:
LKCNK***LV***PL***F***YKTCPAGKNLCYKMFMVATPKVPVKRGCIDVCPKSSLLVKYVC
CNTDRCN
Chain B:
L***K***CNKLV***PL***FYKTCPAGKNLCYKMFMVATP***K***VPVKRGCIDVCP***K***SSLLVKYVC
CNTDRCN
Chain C:
LKCN***KL***VP***LFYK***TCPAGKNL***C***YKMFMVATP***K***VPV***K******RGC***IDVCP***K***SSLLVKYV
CCNTDRCN

Fig. 1. The secondary structure of CTXA3 along with its residues with $P_i > 1$. (The residues with $P_i > 1$ are highlighted in bold and italic formats. Red: loop; green: strand.)

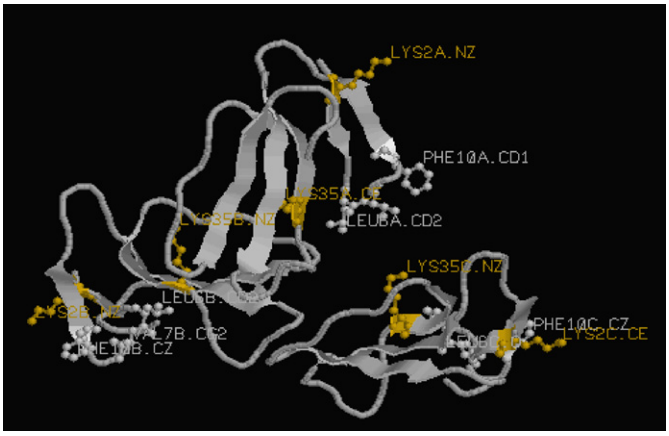

Fig. 2. The pictorial representation of CTXA3 bearing the residues with $P_i > 1$.

SDS, which is also in accord with the experimental reports (Forouhar et al., 2003).

Reports from experimental work point out that the residues involved in the interaction with SDS are situated on the external and internal edges of CTXA3 monomers. The pictorial representation of CTXA3 bearing the residues with $P_i > 1$, which is confirmed by the experimental reports, is shown in Fig. 2.

2.2. Lysozyme

Lysozyme is a known protein with an available high-resolution X-ray diffraction pattern of its crystalline form. Salem et al. (2006) investigated the interaction between TOU and lysozyme by X-ray diffraction. They specified the residues that interact with TOU and pointed out the role of tryptophan residue involved in this interaction.

In the present work, the P_i values for the residues of lysozyme were calculated and are listed in Table 4. The values of $P_i > 1$ are represented in Table 3. All of the residues becoming involved in the interaction with TOU, which have been reported by Prange and co-workers, can be seen among those listed in Table 3. As can be seen, the maximum value of P_i corresponds to trp28. This point is also confirmed by the experimental fact.

2.3. BCA

Bovine carbonic anhydrase is a globular protein with 259 residues and the molecular mass of 30,000. The interaction of this enzyme with SDS has been studied by Whiteside and co-workers (Gudiksen et al., 2006, 2004).

In this work, the interaction of this enzyme with SDS has been studied in terms of ligand binding, which had not been done so far. The calculated values of P_i for the residues of BCA are listed in Table 5 and those with $P_i > 1$ in Table 3. According to these tables the residues effective in interaction with SDS are at first the positive residues of BCA, although the hydrophobic residues are also found in Table 3. The cationic and hydrophobic residues included in Table 3 indicate that SDS will approach the protein molecule from both the hydrophilic head and the hydrophobic tail, as can be expected on the basis of the approach in which the ligand is treated as an amphipathic molecule. These results are in agreement with the amphipathic nature of SDS. Furthermore, these results confirm the idea that in the process of interaction of globular proteins with SDS, the cationic residue on the surface of proteins interacts with SDS initially, followed by the other residues (Jones, 1992; Ding et al., 2007).

Table 4
Calculated P_i values for the lysozyme residues

Res#	P_i	Res#	P_i	Res#	P_i
1	0.20894	51	0.00039	101	3.7933
2	0.82311	52	2.11875	102	0.59884
3	1.23183	53	1.32161	103	1.52303
4	0.40518	54	0.05083	104	0.13558
5	0.24344	55	0.2496	105	0.47114
6	0.56025	56	1.26941	106	1.3259
7	2.59168	57	2.10187	107	0.44412
8	0.01785	58	0.00016	108	3.39409
9	0.16384	59	1.71657	109	0.37049
10	0.21222	60	0.00027	110	0.27997
11	0.56996	61	0.11737	111	1.49379
12	0.18567	62	3.23317	112	1.10405
13	0.64426	63	2.172	113	1.62691
14	0.95116	64	0.00133	114	0.65236
15	0.79543	65	2.6261	115	0.22087
16	0.49507	66	1.30083	116	0.97728
17	1.24364	67	0.99467	117	0.60539
18	1.96606	68	0.97182	118	1.00823
19	1.57012	69	0.86665	119	1.65003
20	1.55008	70	0.39395	120	0.27054
21	0.53518	71	0.38031	121	1.54349
22	0.75914	72	1.45754	122	0.53635
23	3.04769	73	0.68296	123	1.37275
24	0.96305	74	1.48424	124	0.56906
25	0.92957	75	0.92337	125	0.7239
26	0.09765	76	0.22925	126	0.39056
27	0.112	77	2.75786	127	0.72779
28	5.97992	78	0.30137	128	1.23819
29	0.02362	79	0.35279	129	3.22752
30	0.08935	80	0.34238		
31	0.45083	81	1.40464		
32	0.53382	82	0.70129		
33	0.26594	83	0.08475		
34	1.87488	84	1.28274		
35	3.58826	85	1.09399		
36	0.96827	86	1.0423		
37	2.16296	87	2.74414		
38	0.77414	88	0.68203		
39	0.88146	89	0.96925		
40	0.33482	90	0.39099		
41	1.72948	91	0.00702		
42	0.46669	92	0.0152		
43	0.82661	93	0.91311		
44	3.72617	94	0.0941		
45	1.08347	95	0.00947		
46	2.32216	96	0.11009		
47	0.98153	97	0.32336		
48	0.83184	98	0.19737		
49	0.19156	99	0.10057		
50	0.40187	100	0.77395		

A comparison between the results calculated in this work and those of experimental findings shows that the number of residues with $P_i > 1$ is greater than that of the corresponding residues in the experimental information (Forouhar et al., 2003; Salem et al., 2006). This disagreement may be attributable to the difference between the feature of MD simulation and X-ray diffraction. Despite the fact that in the X-ray diffraction procedure the sample is allowed to reach the equilibrium state, the meticulousness and sensitivity of crystallization processes may cause the natural structure to alter. In this work, following references Miamoto and Kollman (1992), Psachoulia et al. (2006) and Bond et al. (2004), when an atom of a ligand has approached a distance $\leqslant 0.4$ nm from an atom of a residue it is considered as a bond formation, whereas in the experimental work the maximum of this range is lower than 0.4 nm. This discrepancy may also contribute to the above disagreement. It should be noted that in dealing with the electrostatic interactions the distance of 0.4 nm will become a basic parameter.

Table 5
Calculated P_i values for the BCA residues

Res#	P_i	Res#	P_i	Res#	P_i	Res#	P_i	Res#	P_i
1	3.76057	51	0	101	2.12308	151	0	201	1.87982
2	0.95115	52	0.00012	102	0.42513	152	3.33987	202	0.39693
3	0.12513	53	0.3635	103	0	153	0.00932	203	0.16497
4	2.71843	54	1.04168	104	0	154	0.7369	204	0.00163
5	0.01386	55	8.20783	105	0.35721	155	0.74319	205	0
6	2.36903	56	0.0339	106	0	156	0.01165	206	0.01596
7	0.96898	57	0.37923	107	0	157	10.0436	207	0.3282
8	13.2885	58	0.07678	108	0	158	0.10101	208	0
9	0.56296	59	0.58905	109	0.08179	159	0.00175	209	0
10	0.20598	60	0.47779	110	0.60047	160	1.01523	210	0
11	0.0028	61	3.03311	111	0.03763	161	1.59601	211	0.00035
12	0.82754	62	0.07375	112	0.59255	162	0.04555	212	0
13	0.28835	63	0.27367	113	0	163	0.04707	213	0
14	0.02528	64	0.01526	114	0	164	1.159	214	0.49084
15	9.97802	65	0.63624	115	0	165	0.01351	215	0.00151
16	6.15756	66	0.92319	116	0	166	0.03087	216	0.01223
17	0.32831	67	0.10823	117	0.4574	167	0.10089	217	0
18	0.19701	68	0.00105	118	0.00175	168	0.00804	218	2.42064
19	9.38232	69	9.69095	119	0.00396	169	3.68205	219	0.41068
20	3.72201	70	0	120	0	170	10.4697	220	0
21	5.72591	71	0	121	0	171	2.43975	221	0.00012
22	4.69704	72	0	122	0	172	1.41228	222	0.73888
23	3.05163	73	0.00885	123	0	173	1.32595	223	0.08342
24	0.49259	74	0	124	0	174	2.81816	224	0.32738
25	0.0113	75	0	125	0.20435	175	0.78863	225	2.25928
26	0.13748	76	0	126	0.54699	176	0.06757	226	0.2788
27	0.79364	77	0	127	0.04218	177	8.81506	227	0.00478
28	1.27877	78	0	128	0.00268	178	5.36148	228	0.06186
29	0.07969	79	0	129	0.75869	179	1.14863	229	0.05662
30	0	80	0	130	0.29534	180	0.2668	230	1.95893
31	0.00396	81	0	131	0.02039	181	6.87127	231	1.86409
32	0	82	0.00163	132	0	182	0.95779	232	3.53909
33	0.0254	83	0.00571	133	0.0395	183	0	233	1.01045
34	0.00012	84	0	134	0.86284	184	0	234	1.63003
35	4.92307	85	0	135	0.26657	185	0.00012	235	0.54303
36	1.92071	86	0	136	0.02307	186	0	236	0.96408
37	0.1059	87	0	137	0.01305	187	0.01689	237	0.37165
38	0.57868	88	0	138	0	188	0	238	2.05493
39	0.76603	89	0	139	0	189	0	239	0.00315
40	0.11487	90	0.01445	140	0	190	0	240	1.3891
41	0.45414	91	1.54289	141	0	191	0.00128	241	0.34707
42	0.05161	92	1.59345	142	0	192	0.00047	242	0.00093
43	0.08808	93	0.14691	143	0	193	0.02621	243	0.34241
44	0.0833	94	0.80855	144	0.00035	194	0	244	7.01994
45	0.04765	95	0.00221	145	0	195	1.60545	245	0.14773
46	0	96	0.06105	146	0.11604	196	1.27387	246	0.0028
47	0	97	0	147	0	197	0.82742	247	0.05289
48	0	98	1.91675	148	0.00023	198	0.90362	248	0.01491
49	0	99	1.7046	149	0	199	0.77733	249	0.35138
50	0	100	7.87066	150	0	200	0.78152	250	11.0573
								251	4.88241
								252	2.18658
								253	1.06894
								254	1.8705
								255	0.47301
								256	0.00047
								257	0.54
								258	0.02586
								259	0.92727

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