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Stoichiometry of Potassium Channel Opener Action

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ABSTRACT

Potassium channel openers (KCOs; e.g., P1075, pinacidil) exert their effects on excitable cells by opening ATP-sensitive potassium channels. These channels are heteromultimers composed with a 4:4 stoichiometry of an inwardly rectifying K^+ channel subunit plus a regulatory subunit comprising the receptor sites for hypoglycemic sulfonylureas and KCOs (a sulfonylurea receptor). To elucidate stoichiometry of KCO action, we analyzed P1075 sensitivity of channels coassembled from sulfonylurea receptor isoforms with high or low P1075 affinity. Concentration activation curves for cDNA ratios of 1:1 or 1:10 resembled those for channel opening resulting from interaction with a single site, whereas models for activation requiring occupation of two, three, or four sites were incongruous. We conclude KCO-induced channel activation to be mediated by interaction with a single binding site per tetradimeric complex.

Potassium channel openers (KCOs) comprise a structurally diverse group of drugs with a broad spectrum of potential therapeutic applications (e.g., hypoglycemia, hypertension, arrhythmias, angina pectoris, asthma) (Lawson, 1996). These drugs (e.g., P1075, pinacidil, levcromakalim, diazoxide) exert their effects on secretory cells, neurons, vascular and nonvascular smooth muscle, and cardiac and skeletal muscle by opening ATP-sensitive potassium channels (K_{ATP} channels), thus shifting the membrane potential toward the reversal potential for potassium and reducing cellular electrical activity (Edwards and Weston, 1993).

Recent progress resulted in cloning of K_{ATP} channels and elucidation of their subunit composition (for review, see Aguilar-Bryan et al., 1998). These channels are assembled with a tetradimeric stoichiometry [sulfonylurea receptor (SUR)/$K_{IR}6.x]_4$ from two structurally distinct subunits, an inwardly-rectifying potassium channel subunit ($K_{IR}6.1$ or $K_{IR}6.2$) forming the pore and a regulatory subunit, an SUR belonging to the ATP-binding cassette superfamily with multiple transmembrane domains, and two nucleotide-binding folds (Aguilar-Bryan et al., 1995; Inagaki et al., 1995, 1996, 1997; Isomoto et al., 1996; Clement et al., 1997; Shyng and Nichols, 1997; Yamada et al., 1997).

Three isoforms of SURs have been cloned, SUR1 and two splice products of a single gene, SUR2A and SUR2B, differing only in their C-terminal 42 to 45 amino acids (Aguilar-Bryan et al., 1995; Chutkow et al., 1996; Inagaki et al., 1996; Isomoto et al., 1996). SUR1/$K_{IR}6.2$ have been proposed to reconstitute the neuronal/pancreatic β-cell (Inagaki et al., 1995), SUR2A/$K_{IR}6.2$ the cardiac (Inagaki et al., 1996; Babenko et al., 1998; Okuyama et al., 1998), and SUR2B/$K_{IR}6.1$ (or $K_{IR}6.2$) the vascular smooth muscle-type K_{ATP} channels (Isomoto et al., 1996; Yamada et al., 1997; Hambrock et al., 1998; Schwanstecher et al., 1998).

Notably, diversity of SURs confers tissue-specific pharmacology, with SUR2 isoforms imparting high sensitivity to KCOs and low sensitivity to sulfonylureas, and SUR1 mediating inverse sensitivities (Inagaki et al., 1995, 1996; Isomoto et al., 1996; Gribble et al., 1998; Schwanstecher et al., 1998; Dörschner et al., 1999; Uhde et al., 1999).

Unraveling the mechanisms involved in control of K_{ATP} activity by SURs is of key importance for understanding molecular pharmacology of these channels. To elucidate stoichiometry of KCO action, we analyzed P1075 sensitivity of channels coassembled from SUR1 and a chimeric construct with high KCO affinity. Concentration-activation curves resembled those expected for channel opening by interaction with a single site.

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ABBREVIATIONS: KCO, potassium channel opener; K_{ATP} channel, ATP-sensitive potassium channel; SUR, sulfonylurea receptor; K_{IR}, inwardly rectifying K^+ channel; DMEM, Dulbecco's modified Eagle's medium.

Experimental Procedures

Materials and Solutions. [^{3}H]P1075 (specific activity 116 Ci mmol^{-1}) was purchased from Amersham Pharmacia Biotech (Freiburg, Germany). All other chemicals and drugs were obtained from the sources described elsewhere (Schwanstecher et al., 1992, 1994, 1998). Stock solutions of drugs were prepared in KOH (50 mM) or dimethyl sulfoxide with a final solvent concentration in the media <1%.

Molecular Biology. SUR1-2 (see *Results* and Fig. 1A; Uhde et al., 1999) was constructed with standard molecular biology techniques comprising amino acids 1 to 1091 and 1358 to 1582 from hamster SUR1 (GenBank accession no. A56248) and 1059 to 1320 from rat SUR2B (GenBank accession no. AF087838). The chimera was subcloned into the pECE vector (Aguilar-Bryan et al., 1995) and sequenced to verify the construct and polymerase chain reaction fidelity before transfection.

Binding Experiments. Transfections and membrane preparations were performed as described (Schwanstecher et al., 1992, 1998). Briefly, COS-7 cells cultured in Dulbecco's modified Eagle's medium (DMEM)-HG (10 mM glucose), supplemented with 10% fetal calf serum, were plated at a density of 5×10^5 cells/dish (94 mm) and allowed to attach overnight. Two hundred micrograms of pECE-SUR complementary DNA was used to transfect 10 plates. For transfection, the cells were incubated 4 h in a Tris-buffered saline containing DNA (5–10 μg/ml) plus DEAE-dextran (1 mg/ml), 2 min in HEPES-buffered salt solution plus dimethyl sulfoxide (10%), and 4 h in DMEM-HG plus chloroquine (100 μM). Cells were then returned to DMEM-HG plus 10% fetal calf serum. Membranes were prepared 60- to 72-h post-transfection as described in Schwanstecher et al. (1992). For binding experiments, resuspended membranes (final protein concentration 5–50 μg/ml) were incubated in Tris-buffer (50 mM; pH 7.4) containing [^{3}H]P1075 (final concentration 3 nM, nonspecific binding defined by 100 μM pinacidil) and unlabelled P1075 or glipizide as indicated in Fig. 1A. The free Mg^{2+} concentration was kept close to 0.7 mM. MgATP (0.1 mM) was added to the incubation media to enable KCO binding (Schwanstecher et al., 1998). Incubations were carried out for 1 h at room temperature and were terminated by rapid filtration through Whatman GF/B filters.

Electrophysiology. Transfections were performed as described above with the following modification. COS-7 cells were plated at a density of 8×10^4 cells/dish (35 mm). 20 μg of pECE-SUR complementary DNA, and 20 μg of pECE-mouse $K_{IR}6.2$ complementary DNA (GenBank accession no. D50581) were mixed and used to transfect six 35-mm plates. Experiments in the inside-out configuration of the patch clamp technique were performed 1 or 2 days after transfection at room temperature as described previously (Schwanstecher et al., 1994). Membrane patches were clamped at −50 mV. The intracellular bath solution contained 140 mM KCl, 2 mM $CaCl_2$, 0.7 mM free Mg^{2+}, 10 mM ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid, 5 mM HEPES (pH 7.3), and the pipette solution 146 mM KCl, 2.6 mM $CaCl_2$, 1.2 mM $MgCl_2$, and 10 mM HEPES (pH 7.4). ADP (0.3 mM) enhances maximal sulfonylurea-induced inhibition of SUR1/$K_{IR}6.2$ channels. It was added to the bath solution to facilitate analysis of glipizide-induced inhibition of channel activity (Fig. 1B; Dörschner et al., 1999). For registration of concentration-response curves (Fig. 1E) patches were chosen with little "run-down" over the measuring period and drug effects were corrected for this loss of channel activity with linear interpolation. Artifacts due to incomplete drug washout or slow reversibility were excluded by making sure that cumulative experiments with stepwise increase or decrease of the drug concentration yielded identical EC_{50} values and slope factors. Channel activity (A) was defined as the product of the number of functional channels (n) and the probability of the channels being in the open state (p). A was calculated by dividing the mean current (I) by the single-channel current amplitude (i). Density of K_{ATP} channels per patch ranged from 10 to 100. Varying channel densities did not affect EC_{50} values, maximal activity (A_{max}) or Hill coefficients. For SUR1-2/SUR1 cDNA ratios of 1:10 (Fig. 1, D and E) only patches with >70 channels were chosen to attain an acceptable frequency of each channel subtype.

Data. Data analysis, including calculation of K_d values from IC_{50} values, and statistics were performed as described (Schwanstecher et al., 1992, 1994). Results are shown as means ± S.E. (n = 3–16).

Theoretical frequencies (P_k) of channel subtypes resulting from coexpression of SUR1-2 (H) and SUR1 (L; Fig. 1C) were calculated with the binomial distribution assuming random assembly:

$$P_k = \binom{4}{k} \cdot P^k \cdot (1 - P)^{4-k}$$

where 4 is the total number of SUR subunits per channel, k the number (0–4) of SUR1-2 subunits in the particular channel subtype, and P or (1 − P) the probability to be incorporated in any of the four positions for SUR1-2 or SUR1, respectively. Theoretical channel activity in the presence of a given concentration of test drug (c) was calculated assuming ordering of the subunits not to affect drug action as follows:

(i) one site model: $\sum_{k=0}^{4} P_k \cdot (1 - (1 - b_H)^k (1 - b_L)^{(4-k)})$

(ii) two-site model: $\sum_{k=0}^{4} P_k \cdot (1 - (1 - b_H)^k (1 - b_L)^{(4-k)}$

$- k \cdot b_H (1 - b_H)^{(k-1)} (1 - b_L)^{(4-k)} - (4 - k) \cdot b_L \cdot (1 - b_H)^k (1 - b_L)^{(3-k)})$

(iii) three-site model: $\sum_{k=0}^{4} P_k \cdot (b_H{}^k \cdot b_L{}^{(4-k)}$

$+ k \cdot (1 - b_H) \cdot b_H{}^{(k-1)} \cdot b_L{}^{(4-k)} + (4 - k) \cdot (1 - b_L) \cdot b_H{}^k \cdot b_L{}^{(3-k)})$

(iv) four-site model: $\sum_{k=0}^{4} P_k \cdot b_H{}^k \cdot b_L{}^{(4-k)}$

where P_k and k are defined as described above, b_H or b_L is the probability of P1075 binding at the concentration c to SUR1-2 or SUR1, respectively, and (4 − k) the number of SUR1 subunits in the particular channel subtype. The values for b_H and b_L were calculated from P1075 concentration-activation curves (Fig. 1E; SUR1-2, EC_{50} = 0.63 ± 0.22 μM; SUR1, estimated EC_{50} = 5.9 mM) assuming binding to be noncooperative (Hill coefficient = 1, Fig. 1A; Schwanstecher et al., 1998) and EC_{50}/K_d ratios of 0.17, 0.62, 1.62, or 5.75 for the 1, 2, 3, or 4-site model, respectively (Dörschner et al., 1999).

Results

The pharmacological hallmark of SUR2B is its high affinity for KCOs, the K_d for P1075 (rat, 11 ± 2 nM; Hill coefficient = 1.01; n = 4) being ~100,000-fold lower than that of SUR1 (hamster, 1.02 mM; Schwanstecher et al., 1998; Fig. 1A). To elucidate stoichiometry of KCO action, we substituted the regulatory domain of SUR1 within the second set of transmembrane domains (K1092-S1357; Uhde et al., 1999) with the corresponding domain of SUR2B (T1059-N1320), thus yielding a SUR1-based construct (SUR1-2; Fig. 1A) with high affinity for P1075 (K_d = 0.17 ± 0.03 μM; Hill coefficient = 0.98; n = 4). High KCO affinity of this construct was paralleled by high P1075 sensitivity of channels transiently reconstituted with $K_{IR}6.2$ in COS-7 cells (EC_{50} value for SUR1-2/$K_{IR}6.2$ channels = 0.63 ± 0.22 μM; Fig. 1, D and E).

Coexpression of SUR1-2 with wild-type SUR1 (cDNA ratio

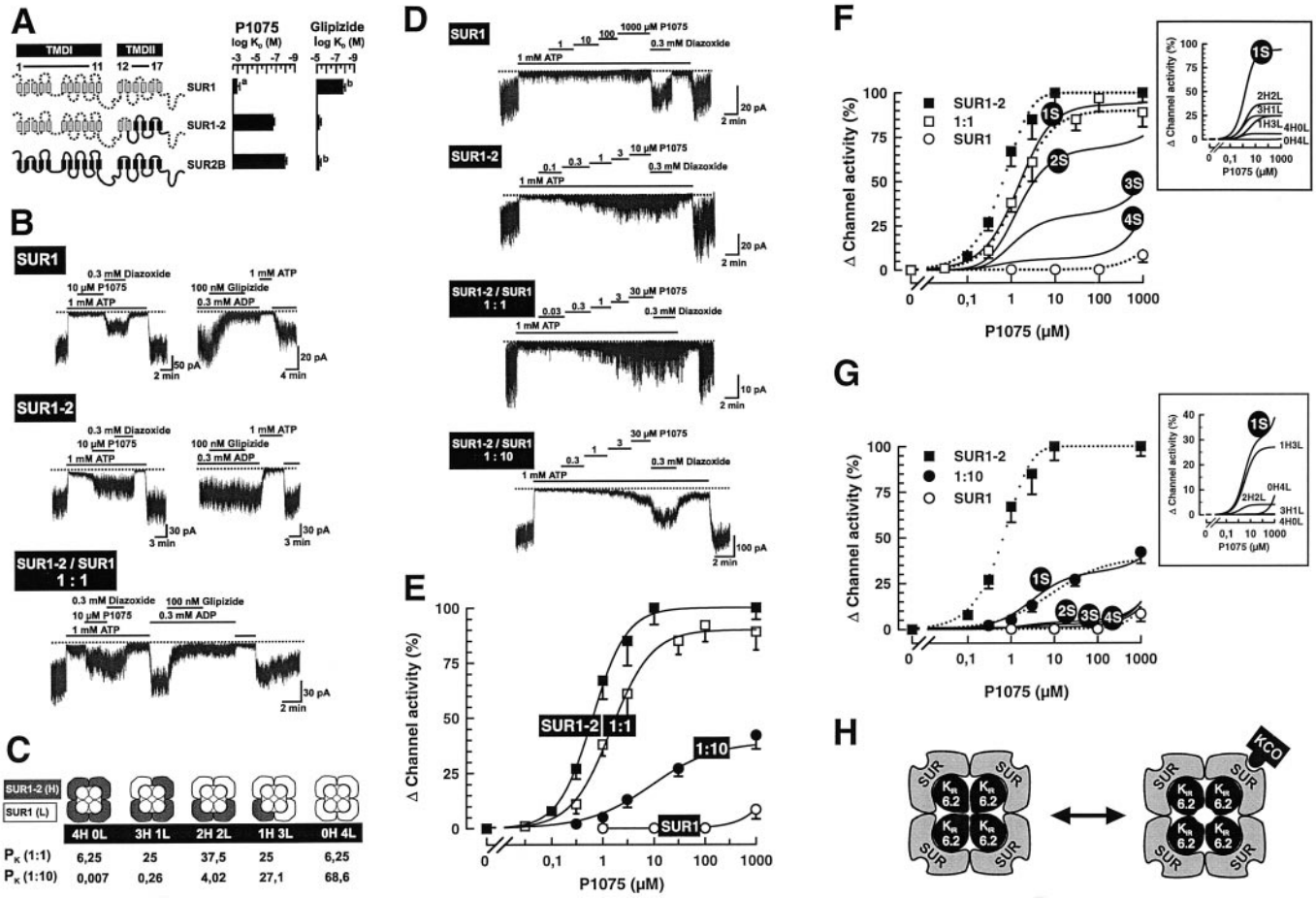

Fig. 1. Stoichiometry of KCO-induced K_{ATP} channel activation. A, P1075 affinity of SUR1-2 is 6000-fold higher than that of wild-type SUR1. Schemata of SUR isoforms are shown on the left, assuming 17 transmembrane domains (Tusnady et al., 1997; TMDI or TMDII, first (1–11) or second (12–17) set of transmembrane domains), dissociation constants (K_d values) for binding of P1075 or glipizide on the right part of the figure. Displacement of [^{3}H]P1075 (3 nM) by unlabelled P1075 or glipizide was assessed with membranes from COS-7 cells transiently expressing wild-type SUR1, SUR1-2, or wild-type SUR2B. K_d values are shown as means ± S.E. calculated from half-maximally inhibitory concentrations (IC_{50} values) of n = 4 to 6 independent displacement curves. K_d values and Hill coefficients were as follows: SUR1 (P1075, 1.02 ± 0.07 mM, 0.91; glipizide 17 ± 3 nM, 0.94), SUR1-2 (P1075, 0.17 ± 0.03 μM, 0.98; glipizide 7.2 ± 0.4 μM, 0.93), SUR2B (P1075, 11 ± 2 nM, 1.01; glipizide 6.1 ± 0.3 μM, 0.99). Values taken from Schwanstecher et al. (1998) (a) or Dörschner et al. (1999) (b). B, coexpression of SUR1-2 and SUR1 (cDNA ratio 1:1) yields channels with high P1075 and glipizide sensitivity. Channels were reconstituted in COS-7 cells by expression of $K_{IR}6.2$ with SUR1, SUR1-2, or SUR1 plus SUR1-2 as indicated. Representative currents recorded at −50 mV from inside-out patches exposed to drugs and nucleotides as indicated by the lines above the records. Diazoxide (0.3 mM) was added to define maximal KCO-induced channel activity (Inagaki et al., 1995; Schwanstecher et al., 1998). Inward currents are shown as downward deflections. C, different species of tetradimeric channels predicted from random assembly. Probabilities of formation (P_k) are given in percentages for SUR1-2/SUR1 ratios of 1:1 and 1:10 (see *Experimental Procedures*). D, P1075 sensitivity of channels reconstituted with $K_{IR}6.2$ [see (B)]. E, potencies of P1075 to open channels reconstituted with SUR1-2 and SUR1. Channel activation was recorded in inside-out patches as shown in (D). Data (n = 4–5) are expressed as percentage of channel activation induced by 0.3 mM diazoxide [see (B) and (D)]. EC_{50} values, maximal activity (A_{max}) and Hill coefficients are as follows: 0.63 ± 0.22 μM, 100%, 1.31 (SUR1-2, ■); 1.5 ± 0.5 μM, 91%, 1.11 (SUR1-2/SUR1, cDNA ratio of 1:1, □); 8.8 ± 1.3 μM, 39%, 0.70 (SUR1-2/SUR1, cDNA ratio of 1:10, ●). F and G, P1075-induced K_{ATP} channel activation is mediated by occupation of a single site. Theoretical concentration-activation curves were constructed assuming 1) a SUR1-2/SUR1 ratio of 1:1 (F) or 1:10 (G), resulting in a distribution of channel species as indicated in (C), and 2) channel activation induced by occupation of one (1S), two (2S), three (3S), or four (4S) binding sites per channel (see *Experimental Procedures*). Measured curves shown in (E) were included to facilitate comparison (dotted lines). Insets, the theoretical curves are the sum of five functions that correspond to the different species of channels predicted from random assembly as shown in (C) (see *Experimental Procedures*). Individual functions shown for the one-site model (1S) and SUR1-2/SUR1-ratios of 1:1 (F) or 1:10 (G), with the channel type indicated beside each curve [see (C)]. H, occupation of a single KCO site per tetradimeric complex induces channel activation.

1:1) yielded a P1075 sensitivity (EC_{50} = 1.5 ± 0.5 μM) similar to that obtained with SUR1-2 alone, but much higher than that of SUR1/$K_{IR}6.2$ channels (EC_{50} value > 1 mM; Fig. 1, D and E), suggesting P1075 sensitivity of coassembled channels to be mainly determined by SUR1-2. Consistent with that idea, the data coincided well with the theoretical curve for channel activation induced by binding to one and any of the four KCO receptor sites per channel complex, whereas the curves for activation requiring occupation of two, three, or four sites were incongruous (Fig. 1F). Analysis of P1075 sensitivity with reduced expression of SUR1-2 (cDNA ratio SUR1-2/SUR1 = 1:10) confirmed this result (Fig. 1, D, E, and G).

Theoretical concentration response curves (Fig. 1, F and G) were constructed assuming both SUR isoforms to distribute randomly in tetradimeric channel composition (Fig. 1C). Validity of this assumption is confirmed by the following findings: 1) Although 10 μM P1075 does not show any effect on the open probability of SUR1/$K_{IR}6.2$ channels (Fig. 1B, top), the same concentration induces maximal activation of channels reconstituted with SUR1-2 (Fig. 1B, middle). 2) However, 100 nM glipizide strongly reduces activity of SUR1/

$K_{IR}6.2$ channels (Fig. 1B, top) without having any impact on channels with SUR1-2 (Fig. 1B, middle; Uhde et al., 1999). 3) The majority of the channels (>90%; $n = 5$) reconstituted with cDNA ratios of 1:1 showed both high P1075 and glipizide sensitivity (Fig. 1B, bottom). These findings demonstrate high P1075 or glipizide potency to be indicative of SUR1-2 or SUR1, respectively, and imply both isoforms to be part of almost all channels expressed from a 1:1 cDNA mixture. This conclusion is consistent with 94% of the channels holding at least one receptor of a type as predicted from random distribution (Fig. 1C).

Controls revealed wild-type SUR1 and wild-type SUR2B not to coassemble with random distribution to form functional K_{ATP} channels (data not shown), excluding use of SUR2B instead of SUR1-2 in this study.

Discussion

Our results indicate that one SUR subunit per tetradimeric K_{ATP} complex confers high KCO sensitivity, implying occupation of a single site to be sufficient for channel activation (Fig. 1H). This conclusion is based on P1075 action on channels originating from coexpression of wild-type SUR1 with a gain of KCO affinity chimera (SUR1-2; Fig. 1). Concentration-activation curves for cDNA ratios of 1:1 or 1:10 resembled those expected for channel opening resulting from interaction with a single site (one-site model).

However, does occupation of additional sites induce stabilization of the open state? Results with reduced expression of SUR1-2 (1:10 ratio) clearly argue against this idea. In these experiments, random distribution suggests channel species with two or more SUR1-2 type receptors to represent <4.5% of all channels (Fig. 1C). Thus, if full channel activation would require binding to more than one site, P1075-induced activation should be significantly weaker than that predicted from the one-site model (Fig. 1G).

It is yet unknown, whether native K_{ATP} channels comprise mixtures of different wild-type SUR isoforms. However, the one-site model would predict KCO sensitivity of mixed channels to be determined by the receptor with highest affinity. Similarly, mutations in SURs reducing KCO affinity and residing in just one allele would be expected to have a minor effect on drug sensitivity. Albeit, in case of increased affinity, KCO sensitivity of K_{ATP} channels would be anticipated to be markedly enhanced.

Potencies of KCOs to activate SUR2B/$K_{IR}6.2$ channels in inside-out patches are 3.5- to 8-fold lower than affinities of SUR2B measured in crude membrane preparations (Schwanstecher et al., 1998) and consistently we observed a similar EC_{50}/K_d ratio with P1075 for SUR1-2 ($EC_{50} = 0.63\ \mu M$, Fig. 1E; $K_d = 0.17\ \mu M$, Fig. 1A; $EC_{50}/K_d = 3.6$). Recently, we have argued that this rightward shift might be due to drug action requiring occupation of more than one site per channel complex (Schwanstecher et al., 1998). Because this explanation does not hold true, lower potencies presumably reflect reduced KCO affinity of SUR subunits in functional channels.

Controls revealed wild-type SUR1 to confer high glipizide sensitivity to almost all (>90%) channels reconstituted by coexpression with SUR1-2 (cDNA ratio 1:1) (Fig. 1B, bottom). This finding implies, in analogy to KCOs, interaction with one SUR subunit to mediate sulfonylurea action, thus confirming conclusions from a recent article (Dörschner et al., 1999).

The study provides new insight into the molecular mechanisms of drug-induced K_{ATP} channel regulation. We conclude KCO-induced channel activation to be mediated by interaction with a single binding site per tetradimeric complex.

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