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Structural requirements for the modulatory effect of 6-substituted pterins on interleukin 2 receptor binding

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(6*R*)-5,6,7,8-Tetrahydrobiopterin is produced by stimulated human T lymphocytes, and is known to affect various aspects of interleukin-2-directed T cell proliferation. Using an increased apparent affinity of interleukin 2 receptor to interleukin 2 as a measure of activity, this study explores whether other 6-substituted pterins might have the same effect, and what structural features are necessary for activity. Of the compounds tested, only the T-lymphocyte-derived (6*R*)-5,6,7,8-tetrahydrobiopterin was active. The diastereomeric (6*S*)-5,6,7,8-tetrahydrobiopterin was inactive, as were 7,8-dihydrobiopterin, sepiapterin, 5,6,7,8-tetrahydroneopterin, 6,7-dimethyl-5,6,7,8-tetrahydropterin and 6-hydroxymethylpterin. 7,8-Dihydroneopterin and neopterin were also found to be inactive. It follows that neither of these compounds participates in the feedback modulation of IL-2 receptor affinity, although both of them can be detected upon IFN-γ stimulation of human monocytes/macrophages. A computer-based molecular modelling study of (6*R*)-5,6,7,8-tetrahydrobiopterin and (6*R*)-5,6,7,8-tetrahydroneopterin revealed substantial differences in overall shape between the two molecules, with certain features figuring prominently in the low-energy conformers of (6*R*)-5,6,7,8-tetrahydrobiopterin.

Introduction

The de novo biosynthesis of (6*R*)-H_4biopterin [1] begins with GTP. The first step is catalysed by GTP-cyclohydrolase I, and leads to H_2neopterin triphosphate with a D-*erythro* configuration of the side-chain at position 6 (cf. Refs. 1,2). This side-chain configuration becomes inverted during subsequent transformations at the tetrahydropterin level of oxidation, however, so that the biosynthetic end-product, (6*R*)-H_4biopterin, carries an L-*erythro*-dihydroxypropyl side chain at position 6 (Fig. 1). Sepiapterin is considered not to be involved in the de novo biosynthetic pathway but rather to represent a breakdown product of unstable intermediates. H_4Biopterin acts as the cofactor for hydroxylation of the aromatic amino acids phenylalanine, tyrosine and tryptophan [3], for the oxidation of L-arginine during nitric oxide formation [4], and for cleavage of glyceryl ethers [5]. The natural material has the 6*R* configuration, and its 6*S* diastereomer has been shown to be significantly different in its cofactor activity [5]. On the other hand, the difference in biological activity between diastereomers having the L-*erythro* and D-*erythro* configurations of the side chain does not seem to be so important for amino acid hydroxylases [7–9].

Recent evidence has shown that H_4biopterin is also produced during cytokine-directed cell proliferation, including clonal expansion of T cells (cf. Ref. 10), and this lymphocyte-derived H_4biopterin seems to have an additional function, unrelated to its cofactor role described above. The clonal expansion of T cells is known to be controlled by the binding of IL-2 to high-affinity receptors on the cell [11,12], and it now appears that this binding can be affected by H_4biopterin [13]. We have shown that in the murine cytotoxic T cell clone CTLL-2, (6*R*)-H_4biopterin can cause a 50% decrease in the K_d value of IL-2 for its binding sites, reducing the dissociation rate of the ligand [13]. This lead ulti-

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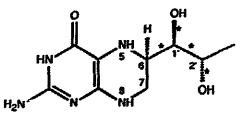
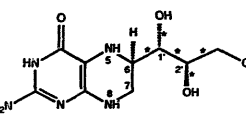
Abbreviations: biopterin, 6-(L-*erythro*-1′,2′-dihydroxypropyl – pterin; H_2biopterin, 7,8-dihydrobiopterin; H_4biopterin, (6*R*)-5,6,7,8-tetrahydrobiopterin; neopterin, 6-(D-*erythro*-1′,2′,3′-trihydroxypropyl)pterin; H_2neopterin, 7,8-dihydroneopterin; H_4neopterin, (6*RS*)-5,6,7,8-tetrahydroneopterin; sepiapterin, 6-(2′-hydroxy-1′-oxopropyl)-7,8-dihydropterin; 6-pyruvcyl-H_4pterin, (6*R*)-(1′,2′-dioxopropyl)-5,6,7,8-tetrahydropterin; IL-2, interleukin 2; IFN-γ, interferon-gamma; HLA, human-leucocyte-associated antigen.



(6*R*)-L-*erythro*-tetrahydrobiopterin

[(6*R*,1'*R*,2'*S*)-6-(1',2'-dihydroxypropyl)-5,6,7,8-tetrahydropterin]



(6*R*)-D-*erythro*-tetrahydroneopterin

[(6*R*,1'*S*,2'*R*)-6-(1',2',3'-trihydroxypropyl)-5,6,7,8-tetrahydropterin]

Fig. 1. Structural formulae and chemical nomenclature for (6*R*)-H_4biopterin (above) and (6*R*)-H_4neopterin (below).

mately to an enhanced proliferation rate of the T cells [14–16]. The objective of the present study was to explore what structural features of the pteridine were important in this new effect on the binding of IL-2 to cells, and to initiate a molecular modelling program which might help to rationalize the results. The pterins used included those which are known to be intermediates of the biosynthetic pathway, and also those which may arise from catabolism. H_2neopterin and neopterin were of special interest because primate monocytes/macrophages are deficient in 6-pyruvoyl-H_4pterin synthase activity. As a result, the biosynthetic pathway in these cells terminates after the initial step, i.e. after the formation of dihydroneopterin triphosphate [17], so that following dephosphorylation of the latter, H_2neopterin and neopterin are formed and released by the cells [17,18]. No physiological role is known for these compounds, however. 6,7-Dimethyl-5,6,7,8-tetrahydropterin was included because it is among the standard pterin substrates for determination of phenylalanine hydroxylase activity [19].

The HTLV-I-infected T-helper cell line MT-2 was used as a test system because it lacks constitutive H_4biopterin synthesis [20]. In order to minimize metabolism of the pterin under study, equilibrium binding of ^{125}I-IL-2 was performed at 4°C.

Materials and Methods

All pterins were obtained from Dr. B. Schircks (Jona, Switzerland). The sources for the medium, fetal calf serum and IL-2 were the same as in Ref. 20. ^{125}I-IL-2 was from NEN (Boston, USA). Remova strips U-form wells were from Dynatech (Denkendorf, Germany).

The origin and methods of long-term cultivation of the MT-2 cells in RPMI 1640 medium, including supplements, are described in Ref. 20. Details concerning equilibrium binding of ^{125}I-IL-2 by the cells are described in Ref. 13. Briefly, the cells were first incubated at 4°C in RPMI 1640 containing 1% bovine serum albumin. They were subsequently transferred to Remova strip U-form wells ($3 \cdot 10^5$ cells/well) and incubated at 4°C for 40 min with serial dilutions of ^{125}I-IL-2 in RPMI plus 1% bovine serum albumin (2:3 dilution steps).

Scatchard analysis of the equilibrium binding data was performed by the LIGAND computer program [21]. Details of its principles are described in Ref. 21 and outlined in Ref. 13. Briefly, the program allows nonspecific binding to be treated as a fitted parameter, evaluates quality of fit, provides calculation of confidence intervals and S.E., and, especially important, allows pooling of multiple experiments. In this study, $n = 3–8$. The program was kindly provided by Dr. P.I. Munson (National Institute of Child Health and Human Development, Bethesda, MD). Freshly prepared stock solutions of each pterin (1 $\mu g\ \mu l^{-1}$) were used for the experiments as described earlier for H_4biopterin [13]. In some experiments, $1 \cdot 10^{-5}$ M 2-mercaptoethanol was included in the stock solution of reduced pterins to yield a final concentration of $1 \cdot 10^{-8}$ M. In these cases, the same concentrations of 2-mercaptoethanol were used in the controls. All reduced pterins were strictly protected from light. Examination of the absorption spectrum showed that more than 80% were still present in their reduced forms with the side chain intact after the time period which was needed for equilibrium binding. Cleavage of the C(6) side-chain is normally accompanied by oxidation of the reduced pyrazine ring [22].

The molecular modelling studies employed the molecular mechanics programme CHARMm [23], and the semi-empirical molecular orbital package AMPAC using the AM1 Hamiltonian [24]. Both were accessed via QUANTA 3.0, supplied by Polygen, Waltham, MA, and implemented on a Silicon Graphics IRIS 4D25TG workstation. Initially, AMPAC was used to energy-minimize a sample conformation of (6*R*)-H_4biopterin. The results were used to assist in the choice of atom types for use with CHARMm, and to assign partial charges for each atom. In particular, they indicated that N5 of the 5,6,7,8-tetrahydropteridine ring system is essentially pyramidal, and therefore needed to be modelled as an 'amino'-type nitrogen with both pseudoaxial and pseudoequatorial orientations possible for the N-H. N8 was found to be more nearly planar.

CHARMm was then employed for conformation searches of (6*R*)-H_4biopterin and, as a comparison, (6*R*)-H_4neopterin (Fig. 1). Four families of structures were considered for each molecule, featuring the C(6) side-chain and N(5)-H in all possible pseudoaxial/pseudoequatorial combinations. Starting conformations for energy-minimization were then generated by rotations about the starred bonds in Fig. 1. After the CHARMm minimization, about 10 low-energy structures were selected from each family and subjected to reminimization using AMPAC, yielding a total of about 40 energy minima for each molecule.

Results and Discussion

Scatchard plots of the data obtained from the equilibrium binding experiments on ^{125}I-IL-2 are shown in Fig. 2. The K_d value of the control batch of MT-2 cells was found to be 82.7 pM IL-2 (95% confidence interval: 78.9–86.5 pM IL-2). In contrast, when (6*R*)-H_4biopterin ($3 \cdot 10^{-7}$ M) was added during the binding period, the K_d value dropped to 41.1 pM IL-2 (95% confidence interval: 39.4–43.0 pM IL-2), showing that this pterin increases the apparent affinity of the cells for IL-2. These experiments demonstrate that MT-2 cells react in the same way as previously found for CTLL-2 cells [13].

For the experiments which tested the effect of other pteridines on IL-2 binding, the control cells originated from a different culture batch (see Ref. 20). They showed a slightly higher K_d value of 95.5 pM IL-2 (95% confidence interval: 94.5–96.0 pM IL-2). With these cells, (6*R*)-H_4biopterin reduced the K_d exactly as described above for the first culture batch. The binding curve obtained in the presence of the epimeric (6*S*)-H_4biopterin was indistinguishable from that of the control cells (data not shown), showing that the modulatory effect of H_4biopterin is restricted to the natural (6*R*) isomer alone. The effect of several other

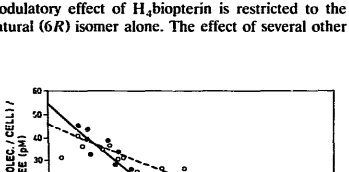


Fig. 2. Scatchard analysis of equilibrium binding of ^{125}I-IL-2 to MT-2 cells. Each data point represents triplicate determinations which were processed by the LIGAND program. Five independent experiments were pooled. (o——o) control cells, K_d = 82.7 pM IL-2; (•——•) with (6*R*)-H_4biopterin ($3 \cdot 10^{-7}$ M), K_d = 41.1 pM IL-2.

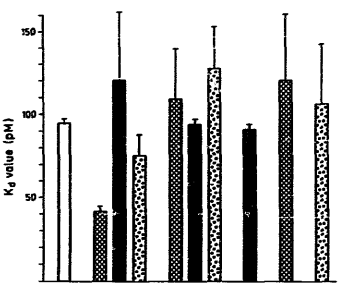


Fig. 3. Modulation of the IL-2 receptor affinity for the ligand by 6-substituted pterins. S.D. $n = 5$–8. Pterins were added at a concentration of $3 \cdot 10^{-7}$ M. C, control; H_4B, H_3biopterin; H_2B, H_2biopterin; B, biopterin; H_3N, H_4neopterin; H_2N, H_2neopterin; N, neopterin; S, sepiapterin; H_2DMP, 6,8-dimethyl-5,6,7,8-tetrahydropterin; HMP, 6-hydroxymethylpterin.

pterins on the K_d value was also investigated. H_2biopterin did not change the K_d value, whereas biopterin itself decreased it slightly. Neopterin, H_2neopterin and H_4neopterin, as well as sepiapterin, all failed to exhibit any modulatory effect. And finally, neither 6-hydroxymethylpterin nor 6,7-dimethyl-5,6,7,8-tetrahydropterin had any effect on the affinity of the IL-2 receptor for its ligand. These results are summarized in Fig. 3. It thus appears that, of all the compounds tested, only (6*R*)-H_4biopterin (and biopterin to a slight extent) was able to influence the binding of IL-2 to its high-affinity receptor on the T cells, and thus to influence the proliferation of these cells. This points to the importance of the 1,2-dihydroxypropyl side-chain at position 6. It also seems that, in order to exhibit maximum activity, the pterin should be in its tetrahydro reduced form, with the 6*R* configuration.

It is important to emphasize that the assay temperature used for all our tests was 4°C. The dissociation constant of a given ligand is normally independent of the temperature. At 4°C, however, any possible metabolic change of the added pterin during the test was minimized, thus ensuring that the results obtained were really due to the added pterin and not to a metabolically derived product. A co-mitogenic effect was previously observed [15] for H_2biopterin and for sepiapterin under experimental conditions which involved incubation at 37°C. The inactivity of these two compounds in the present study, however, would suggest that their previously reported activity was probably due to H_4biopterin, formed metabolically in situ at

37°C from the added H_2biopterin and sepiapterin, as was suggested earlier [15]. The data described above also show that neopterin and H_2neopterin have no role in IL-2 receptor modulation. As already mentioned, these are the pterins produced by primate monocytes/macrophages.

The IL-2 receptors on T cells are known to be associated with class I HLA molecules (cf. Ref. 12), and we have shown (Ziegler et al., unpublished results) that pterin binding sites are on these class I HLA molecules. A possible role for the H_4biopterin in complexing to the HLA molecules is to induce a conformational change in the latter, which in turn could activate IL-2 receptor formation. As an aid to understanding the specificity of the H_4biopterin binding site, and with the long-term goal of designing agonists and/or antagonists for this receptor, we have undertaken a programme of computer-based molecular modelling studies. This allows calculation of the energies of the different possible conformations of a molecule, and can therefore point to those conformations which may be involved in any particular process. There is already one other report in the literature of a molecular modelling study of H_4biopterin [6]. However, we have employed a more rigorous method of calculation which, as discussed below, points to different conclusions.

Our initial work concentrated on (6*R*)-H_4biopterin itself and, as a comparison, its inactive relative (6*R*)-H_4neopterin (Fig. 1). As described under Materials and Methods, we used a 'molecular orbital - molecular mechanics - molecular orbital' sequence of calculations to identify about 40 low-energy conformers for each molecule. In general, the results confirmed our expectation that the molecules are sufficiently flexible to prevent definition of the binding site by this work alone. According to the calculations, (6*R*)-H_4biopterin and (6*R*)-H_4neopterin have 8 and 10 energy minima, respectively, within 1.5 kcal/mol of baseline. When conformers are so close together on the energy scale, it is impossible to say with confidence which ones are likely to be involved in binding. The interpretation is further complicated by the fact that the calculations give gas-phase structures and energies, and so do not take account of the likely environment within the binding site. Bearing these limitations in mind, however, a survey of the low-energy conformers does encourage certain generalizations. Notably, of the eight lowest energy minima of (6*R*)-H_4biopterin referred to above, seven have an axial C(6) side-chain in which the C(1')-OH is antiperiplanar to the C(6)-N(5) bond. These include the 'global miniumum' conformer (ΔH_f = −92.6803 kcal/mol), which is shown as B1 in Fig. 4, in

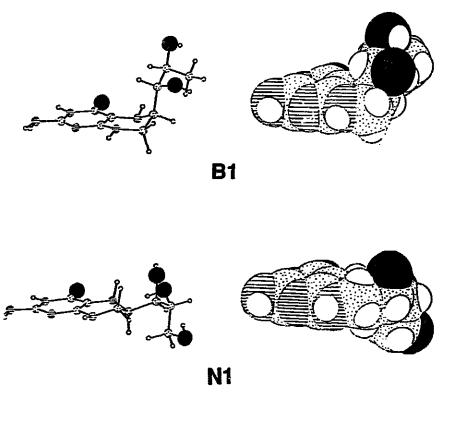


Fig. 4. Global energy minima for (6*R*)-H_4biopterin (B1), and (6*R*)-H_4neopterin (N2), as predicted by the 'molecular orbital - molecular mechanics - molecular orbital' sequence of calculations described in the text.

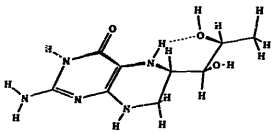


Fig. 5. Global energy minimum for (6*R*)-H_4biopterin derived from molecular mechanics calculations, as reported by Ayling et al. [4].

both bail-and-stick and space-filling modes. All bar one of these seven conformers have an equatorial N(5)-H (as in B1) and all bar one have the same conformation about C(1')-C(2') as found in B1. The latter could thus be a good representative of the 'overall structure' of (6*R*)-H_4biopterin, possibly related to the active conformer. This does not, of course, rule out the one low-energy conformer with an equal (C6) side-chain. For (6*R*)-H_4neopterin, the 6 lowest-energy conformers (within 1.5 kcal/mol of the baseline) all have an equatorial C(6) side-chain with the orientation about the C(6)-C(1') bond, such that the C(1')-OH is antiperiplanar to C(6)-H. The global minimum ($H_f = -140.8365$ kcal/mol) is shown as N1 in Fig. 4, a notable feature being a weak hydrogen bond (2.49 Å) between the C(2')-OH and the N(5) lone pair of electrons.

The report of Ayling et al. already referred to [6] described a molecular mechanics study on (6*R*)- and (6*S*)-H_4biopterin. In contrast to our results, these authors concluded that the lowest energy conformer of (6*R*)-H_4biopterin has a hydrogen bond between N(5)-H and the C(2')-O, the side chain apparently being pseudoequatorial (Fig. 5). We have made a particular effort to locate conformers of this type using our methodology, but have found that the one which most closely mimics Fig. 5 is 2.584 kcal/mol above the baseline. The lowest-energy conformer with an N(5)-H...O-C(2') hydrogen bond is one with a pseudoaxial side-chain, at 1.534 kcal/mol above the baseline. As already described, our calculations are based on a combination of molecular mechanics and molecular orbital methods. The earlier workers mention molecular mechanics as the basis for their calculations but give no other details, and the differences between their conclusions and ours presumably relate to the methods using to calculate the energies.

Acknowledgements

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