J. Biochem. 122, 1040-1045 (1997)

Substrate Specificity for Isomerase Activity of Macrophage Migration Inhibitory Factor and Its Inhibition by Indole Derivatives[1]

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Received for publication, July 4, 1997

Macrophage migration inhibitory factor (MIF) was discovered as a cytokine that inhibits random migration of macrophages and concentrates them at inflammatory loci. We recently reported the tertiary structure of MIF, and revealed its similarity to that of 5-carboxymethyl-2-hydroxymuconate isomerase. Moreover, MIF was found to have isomerase activity converting D-dopachrome, a stereoisomer of naturally-occurring L-dopachrome, to 5,6-dihydroxyindole-2-carboxylic acid. In this study, we examined the effects of a series of compounds analogous to D-dopachrome on the enzyme activity to obtain vital information for identification of a natural substrate of MIF. Adrenochrome, lacking a carboxyl group at position 2 of the indolinequinone ring, could not be a substrate. Several indole-ring-containing compounds with a carboxyl group were inhibitory to D-dopachrome isomerase activity, of which indole-3-acrylic acid was the most potent inhibitor, with an inhibitor constant (K_i) of 2.8 mM. 2,3-Indolinedione, which lacks a complete indole ring or a carboxyl group but has carbonyl groups at positions 2 and 3, apparently inhibited the enzyme activity in a competitive or mixed manner with a K_i of 0.9 mM. Taken together, these facts suggest that the 2-carboxyl group of the substrate is essential for interaction with the active site of MIF.

Key words: cytokine, dopachrome, isomerase, macrophage, macrophage migration inhibitory factor.

Macrophage migration inhibitory factor (MIF) was originally discovered as a T lymphocyte-derived cytokine inhibiting migration of macrophages out of capillary tubes (*1, 2*). In addition to its inhibitory action, MIF was found to have a variety of biological functions such as activation of macrophages and enhancement of their tumoricidal and parasiticidal activities (*3-5*). Furthermore, MIF is an anterior pituitary-derived hormone that potentiates lethal endotoxaemia (*6*) and overrides the glucocorticoid-mediated suppression of inflammatory and immune responses (*7*). Recently, it was found to play an essential role in the activation of T lymphocytes after mitogenic or antigenic stimuli (*8*).

We recently succeeded in crystal structure analysis of MIF. This uncovered an unexpected similarity with an isomerase, 5-carboxymethyl-2-hydroxymuconate isomerase (CHMI) from *Escherichia coli*, even though little amino acid sequence homology exists between these two proteins (*9-11*). On the other hand, the amino acid sequence of MIF (*12*) shares homology with D-dopachrome tautomerase (DOPD) (*13*), which catalyzes isomerization of D-dopachrome, a stereoisomer of naturally occurring L-dopachrome, with concomitant decarboxylation to give 5,6-dihydroxyindole (DHI) (*14*). Although the tertiary structure of DOPD has not been reported, these primary and tertiary structural similarities raised the possibility that MIF may have isomerase activity. Indeed, Rorsman *et al.* demonstrated that MIF isolated from bovine eye lens and recombinant human MIF converted D-dopachrome into 5,6-dihydroxyindole-2-carboxylic acid (DHICA) (*15*) (Scheme 1).

The physiological significance of the D-dopachrome isomerase activity exerted by MIF remains to be elucidated because its natural substrate has not yet been found. Thus identification of a true substrate is considered to be crucial for elucidation of the physiological function of MIF in association with D-dopachrome isomerase activity. In this study, we examined the substrate specificity of MIF and inhibitory effects of a series of natural and organic-synthesized chemical compounds on the isomerase activity to obtain information essential for identification of the true substrate, which may be pivotal in understanding the pathophysiological role of MIF.

MATERIALS AND METHODS

Materials—The following materials were obtained from commercial sources. Sephadex G-100 was from Pharmacia (Uppsala, Sweden); *S*-hexylglutathione-linked Sepharose, L-dopa, D-dopa, DL-α-methyldopa, L-dopa methyl ester, 5-hyroxyindole, 5-hyroxyindole-3-acetic acid, L-tryptophan

[1] This work was supported in part by a Grant-in-Aid for Research (No. 07670162) from the Ministry of Education, Science, Sports and Culture of Japan.

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Abbreviations: CHMI, 5-carboxymethyl-2-hydroxymuconate isomerase; DCT, dopachrome tautomerase; DHI, 5,6-dihydroxyindole; DHICA, 5,6-dihydroxyindole-2-carboxylic acid; DOPD, D-dopachrome tautomerase; MIF, macrophage migration inhibitory factor.

methyl ester, D-tryptophan, D-tryptophan methyl ester, 5-hydroxy-L-tryptophan, tryptamine, serotonin, and melatonin were from Sigma (St. Louis, MO, USA); epinephrine was from Merck (Darmstadt, Germany); indole-3-acetic acid, indole-3-acetic acid ethyl ester, indole-3-propionic acid, and indole-3-acrylic acid were from Nacalai Tesque (Kyoto); indole and isopropyl-β-D(−)-thiogalactopyranoside (IPTG) were from Wako (Osaka), and 2,3-indolinedione was from Kanto (Tokyo). DHICA was prepared as previously described (*16*). All other chemicals were of analytical grade.

Purification of MIF—Recombinant rat MIF was purified as previously described (*17*). Briefly, *E. coli* BL21(DE3)-pLysS, transformed with the T7 expression plasmid pET-3a (*18*) containing a cDNA coding for rat MIF, was cultured and expression of MIF protein was induced by IPTG. Cells were disrupted by use of a French pressure cell disrupter at 1,000 psi, then the homogenate was centrifuged at 105,000×*g* for 1 h. From the supernatant, MIF was purified by *S*-hexylglutathione affinity column chromatography followed by Sephadex G-100 gel-filtration column chromatography.

Preparation of Dopachrome and Related Compounds—Solutions of dopachrome were prepared essentially as described (*19*). L- or D-dopa was mixed with a 1.9-fold molar quantity of sodium periodate in 100 mM potassium phosphate buffer (pH 6.5) containing 0.1 mM EDTA. An excess of dopa over stoichiometry (dopa : periodate = 1 : 2) was used to avoid further oxidation of the reaction products by periodate. The dopachrome solutions were prepared immediately before use because of its relative instability. L-Dopachrome methyl ester, DL-α-methyldopachrome, and adrenochrome were prepared by the same procedure from L-dopa methyl ester, DL-α-methyldopa, and epinephrine, respectively.

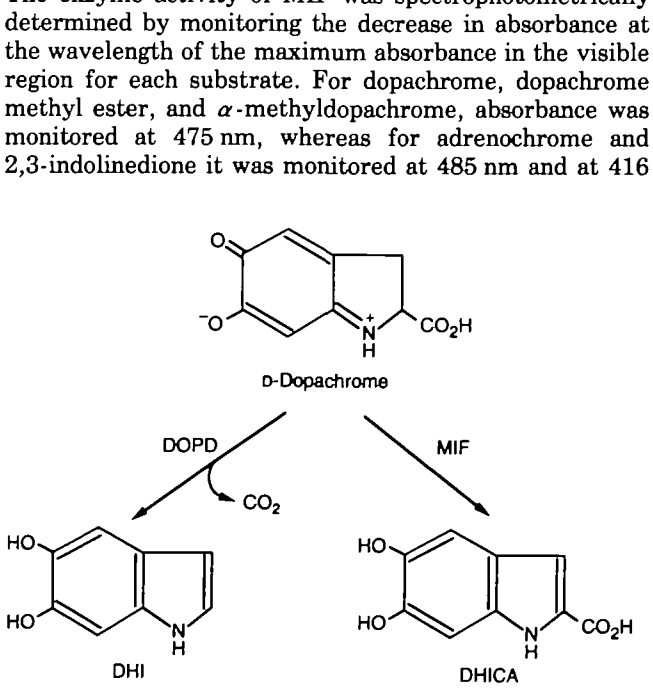
Determination of D-Dopachrome Tautomerase Activity—The enzyme activity of MIF was spectrophotometrically determined by monitoring the decrease in absorbance at the wavelength of the maximum absorbance in the visible region for each substrate. For dopachrome, dopachrome methyl ester, and α-methyldopachrome, absorbance was monitored at 475 nm, whereas for adrenochrome and 2,3-indolinedione it was monitored at 485 nm and at 416 nm, respectively. On the other hand, when 2,3-indolinedione was added as an inhibitor, the wavelength was set at 525 nm to amplify the ratio of the absorbance of dopachrome to that of 2,3-indolinedione. This ratio had the value of 34 at 525 nm but 8.8 at 475 nm.

For inhibition assay, most inhibitors were dissolved in 100 mM potassium phosphate buffer (pH 6.5) containing 0.1 mM EDTA and mixed with dopachrome solutions. For compounds with low solubility, dimethylsulfoxide (DMSO) was added to the reaction mixtures to give a final concentration of 2%. To calculate the rate of enzymatic reaction, the decrease in absorbance in the absence of MIF was subtracted from the value obtained in its presence. Since absorbance decreased linearly for at least 30 s, it was monitored for the initial 20 s, from which the rate of reaction was determined. All assays were carried out at 24°C and repeated at least three times.

RESULTS

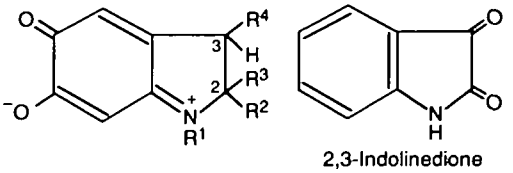
Substrate Specificity—Besides L- and D-dopachromes, we examined L-dopachrome methyl ester, DL-α-methyldopachrome, adrenochrome and 2,3-indolinedione as substrates for MIF. The structures of these compounds are listed in Table I. Enzyme activity for each compound is shown in Table II. In agreement with the data reported by Rorsman *et al.*, no enzymatic activity was observed when L-dopachrome was used as a substrate, whereas its methyl ester was a much more appropriate substrate (about 70-fold) than D-dopachrome (*15*). Adrenochrome, which has a similar chemical structure to D-dopachrome but lacks a 2-carboxyl group, was not converted by MIF to its corresponding indole derivative. Similarly, dopaminochrome, which also lacks this functional group, could not be a substrate (*15*). Thus, it is considered that the 2-carboxyl group of D-dopachrome is essential for substrate recognition.

α-Methyldopachrome has no hydrogen atom linked directly to the carbon atom at position 2 of the indolinequinone ring. As expected, this compound was not isomerized in the presence of MIF. The slow decolorization observed both in the presence and absence of MIF might have been caused by rearrangement of the product of



Scheme 1. **Conversion of D-dopachrome catalyzed by DOPD and by MIF.**

TABLE I. **Structures of possible substrates of MIF.**



Substrate	R^1	R^2	R^3	R^4
Dopachrome	H	CO_2H	H	H
Dopachrome methyl ester	H	CO_2CH_3	H	H
α-Methyldopachrome	H	CO_2H	CH_3	H
Adrenochrome	CH_3	H	H	OH

spontaneous decarboxylation. Another analogue, 2,3-indolinedione, having two oxygen atoms on the contralateral side in comparison with dopachrome, failed to show any measurable decrease in visible absorption at 416 nm. Its conversion to the corresponding dihydroxy compound is unlikely, because 2,3-indolinedione lacks any saturated carbon, and its oxygens are located at the opposite side.

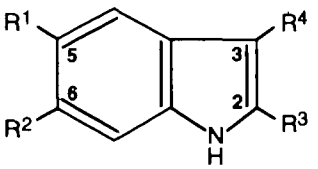
Inhibitory Effect of Indole-Ring-Containing Compounds on Tautomerization of MIF—The chemical structures of the indole derivatives tested as inhibitors of MIF are shown in Table III. Before evaluating their inhibitory activity, we examined the effect of DMSO on the isomerization activity of MIF and found that MIF retained almost its full activity even in the presence of 2% DMSO compared to that in its absence. The inhibition by the indole derivatives is shown in Table IV. The authentic product of the isomerization reaction, DHICA, showed weak inhibitory activity. Unexpectedly, 5-hydroxyindole-2-carboxylic acid, an analogue of DHICA that lacks a hydroxyl group at position 6 of the indole ring, showed more potent inhibitory activity than DHICA. The inhibitor constant (K_i) for 5-hydroxyindole-2-carboxylic acid and other indole-ring-containing compounds such as indole-3-acetic acid, indole-3-acrylic acid are shown in Table V. From these results, it was found that

TABLE II. **Action of MIF on different possible substrates.**

Substrate	[Substrate] (mM)	[MIF] (μg/ml)	Rate of reaction (μM/min)		Activity (μmol/min/mg MIF)
			+MIF	−MIF	
L-Dopachrome	0.5	50	24	22	0.0
L-Dopachrome	0.5	50	214	19	3.9
L-Dopachrome methyl ester	0.5	0.5	262	126	272
DL-α-Methyldopachrome	0.5	50	11	11	0.0
Adrenochrome	0.5	50	7	7	0.0
2,3-Indolinedione	2[a]	50	0	0	0.0

[a]Assayed in the presence of 2% DMSO.

TABLE III. **Structures of possible inhibitors of MIF.**



Inhibitor	R^1	R^2	R^3	R^4
Indole	H	H	H	H
5-Hydroxyindole	OH	H	H	H
Indole-3-acetic acid	H	H	H	CH_2–CO_2H
Indole-3-propionic acid	H	H	H	CH_2CH_2–CO_2H
Indole-3-acrylic acid	H	H	H	CH=CH–CO_2H
Indole-3-acetic acid ethyl ester	H	H	H	CH_2–$CO_2CH_2CH_3$
5-Hydroxyindole-3-acetic acid	OH	H	H	CH_2–CO_2H
5-Hydroxyindole-2-carboxylic acid	OH	H	CO_2H	H
5,6-Dihydroxyindole-2-carboxylic acid	OH	OH	CO_2H	H
Tryptophan	H	H	H	CH_2–$CH(NH_2)$–CO_2H
Tryptophan methyl ester	H	H	H	CH_2–$CH(NH_2)$–CO_2CH_3
5-Hydroxytryptophan	OH	H	H	CH_2–$CH(NH_2)$–CO_2H
Tryptamine	H	H	H	CH_2CH_2–NH_2
Serotonin	OH	H	H	CH_2CH_2–NH_2
Melatonin	OCH_3	H	H	CH_2CH_2–NHCOCH$_3$

TABLE IV. **Inhibition of enzyme activity by indole derivatives.**[a]

Inhibitor	Concn. (mM)	Inhibition (%)
Indole	5[b]	<10
5-Hydroxyindole	5	<10
Indole-3-acetic acid	5	35
Indole-3-propionic acid	5	11
Indole-3-acrylic acid	5	60
Indole-3-acetic acid ethyl ester	2[b]	<10
5-Hydroxyindole-3-acetic acid	5	10
5-Hydroxyindole-2-carboxylic acid	5	38
5,6-Dihydroxyindole-2-carboxylic acid	5	24
L-Tryptophan	5	<10
L-Tryptophan methyl ester	5	<10
D-Tryptophan	5	<10
D-Tryptophan methyl ester	5	<10
5-Hydroxy-L-tryptophan	5	<10
Tryptamine	5	<10
Serotonin	5	<10
Melatonin	2[b]	<10
2,3-Indolinedione	5[b]	81

[a]Assays were carried out with 0.5 mM D-dopachrome solutions containing 25 μg/ml MIF and indicated concentrations of inhibitors. [b]Assayed in the presence of 2% DMSO.

TABLE V. **Inhibitor constants for relatively potent inhibitors.**

Inhibitor	K_i (mM)
Indole-3-acetic acid	8[a]
Indole-3-acrylic acid	2.8[a]
5-Hydroxyindole-2-carboxylic acid	8[a]
2,3-Indolinedione	0.9[b]

[a]Reaction rates were calculated for 0.25 and 0.5 mM D-dopachrome solutions containing 25 μg/ml MIF. K_i values were determined by Dixon plot analysis. [b]Assayed in the presence of 2% DMSO. Analytical conditions are detailed in Fig. 2.

indole-3-acrylic acid was the strongest inhibitor among the indole-ring-containing analogues tested. The other two compounds showed the same order of affinity for MIF as D-dopachrome.

On the other hand, neither L-tryptophan nor its methyl ester was found to have inhibitory activity for D-dopachrome tautomerization, although tryptophan also has an indole ring substituted at position 3 and its side chain contains a carboxyl group. Similar results were obtained for D-tryptophan and its methyl ester. In addition, 5-hydroxy-L-tryptophan and metabolites of L-tryptophan, such as tryptamine, serotonin, and melatonin, failed to inhibit the enzyme activity. It is of note that all the inhibitors except 2,3-indolinedione contain a carboxyl group. 2,3-Indolinedione, which lacks a carboxyl group, contains two carbonyl groups instead. These results indicated that the 2-carboxyl group of D-dopachrome may be critical for substrate or inhibitor recognition by the active site of MIF.

Figure 1 shows a double-reciprocal plot of 2,3-indolinedione for the isomerase activity of MIF. The extrapolated Michaelis constant (K_m) value in the solution containing 2% DMSO was estimated to be greater than 5 mM. From a Dixon plot, the K_i for 2,3-indolinedione was determined to be 0.9 mM (Fig. 2). Three independent sets of experiments suggested competitive or mixed, rather than noncompetitive inhibition. This showed that this compound was a relatively strong inhibitor of MIF. In this context, the

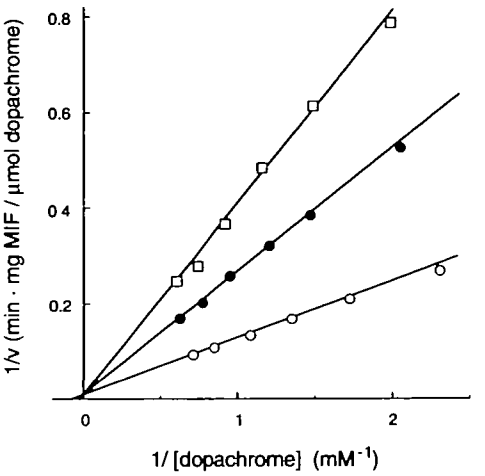


Fig. 1. **Double reciprocal plot of enzyme activity of MIF in the presence and absence of 2,3-indolinedione.** Reaction rates were calculated for D-dopachrome solutions containing 50 μg/ml MIF and 2% DMSO in the absence (○) and in the presence of 1 mM (●) or 2 mM (□) 2,3-indolinedione.

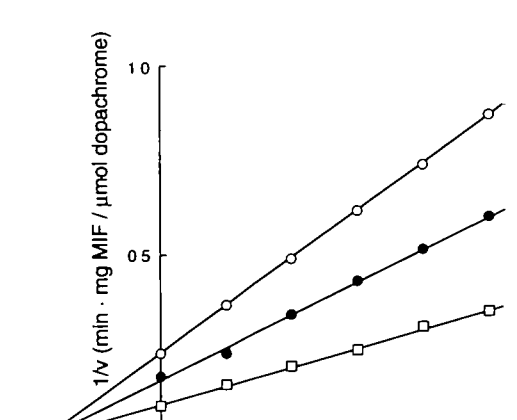


Fig. 2. **Dixon plot of enzyme activity of MIF in the presence of 2,3-indolinedione.** Assays were carried out with 0.5 mM (○), 0.75 mM (●), and 1.25 mM (□) D-dopachrome solutions containing 50 μg/ml MIF, 2% DMSO, and indicated concentrations of 2,3-indolinedione.

chemical structure of 2,3-indolinedione may help to elucidate the structure of the active site of MIF.

DISCUSSION

Dopachrome tautomerase (DCT) catalyzes the conversion of L-dopachrome to DHICA in the biosynthetic pathway of melanin. DCT, also designated tyrosinase-related protein-2 (TRP-2), is a membrane-bound zinc-containing glycoprotein (*20, 21*). On the other hand, MIF is an intracellular soluble protein forming a homotrimeric structure and converts D-dopachrome to DHICA. MIF is present in the cytosol and is extracellularly secreted after stimulation by substances such as lipopolysaccharide (*6*). MIF, in contrast to DCT, does not require a metal ion in order to exert its enzyme activity, and no sequence homology is found

between DCT and MIF.

DCT can convert L-dopachrome methyl ester and L-dopachrome at similar conversion rates (*19*). On the other hand, MIF does not recognize L-dopachrome as a substrate. It is of note that MIF is able to convert D-dopachrome methyl ester and L-dopachrome methyl ester as well as D-dopachrome to each corresponding product (*15*). However, the reaction rate for L- or D-dopachrome methyl ester is much greater than that for D-dopachrome, in contrast to the nearly equal rates of isomerization of L-dopachrome and its methyl ester catalyzed by DCT. As for inhibitors, L-tryptophan is one of the strongest inhibitors of DCT (*19*), while this aromatic amino acid does not suppress D-dopachrome tautomerization activity of MIF.

Despite these distinct properties, several similarities in substrate specificity and spectra of inhibitors were found in this study. Aroca *et al.* showed that analogues of dopachrome lacking the carboxyl group at position 2 were not isomerized by DCT (*19*), as was shown for MIF in the present study. It is also known that DCT is inhibited by some indole derivatives in a manner similar to that demonstrated for MIF in this study. For example, both DHICA and 5-hydroxyindole-2-carboxylic acid have the potential to inhibit isomerization of D-dopachrome catalyzed by either DCT or MIF. These facts may reflect some common characteristics in the mechanisms of substrate recognition of the two enzymes. In this respect, it appears worthwhile to compare their substrate specificities and the chemical structures of the inhibitors of their isomerase activity.

All the substrates and inhibitors found for DCT contain a free or methylated carboxyl group (*19*). This finding is comparable with our results for MIF. In inhibition by substituted indoles, three carbon atoms of the side chain including the carbon atom of the carboxyl group were required for the maximal inhibition of DCT. Hence, it was speculated that the potential of the carboxyl group in this position to maximize inhibition may derive from adopting a location that allows it to mimic the 2-carboxyl group of the authentic substrate. Our finding that indole-3-acetic acid was a more effective inhibitor of MIF than indole-3-propionic acid indicated that a side chain containing two carbon atoms was optimal for inhibition of MIF. This implies that the structures of the active sites of the two enzymes may differ to a relatively great extent, even if they share common features.

It is worthwhile to compare the tertiary structure of MIF with that of CHMI for further understanding of the catalytic mechanism. For CHMI, it has been proposed that two arginine residues interact with two carboxyl groups of the substrate to form the substrate-enzyme complex (*11*). The proposed catalytic center of CHMI is the N-terminal proline residue. Although the active site of MIF has not been clearly identified, it is speculated that its catalytic center may be located around the N-terminal region, because of the presence of a proline residue at its N-terminus (*9, 10*). The proline residue is surrounded by a number of aromatic residues forming a large hydrophobic pocket. Considering the molecular structures of the substrates and inhibitors of MIF, it appears reasonable to consider that the substrate is incorporated in this pocket. The fact that indole-3-acrylic acid is a stronger inhibitor of MIF than indole-3-acetic acid or indole-3-propionic acid, even though the corresponding carboxyl group of indole-3-acrylic acid is located at a position distant from the 2-carboxyl group of D-dopachrome, supports the idea. To explain the unfavorable location, indole-3-acrylic acid has a highly conjugated structure which is likely to contribute to the interaction with the cluster of aromatic residues of the active site.

If the N-terminal pocket comprises the catalytic site of MIF, the carboxyl group of a substrate is suggested to bind to a specific locus in the pocket. There are two candidates for the carboxyl-binding residue, Lys32 juxtapositioned to Pro1, and Arg93 belonging to the adjacent subunit (Fig. 3). In support of this possibility, these residues are conserved in rat DOPD (*9, 13*), and Arg93 is conserved in CHMI in spite of the fact that there is little sequence homology with MIF (*9, 22*). As for the catalytic mechanism, the initial reaction step is speculated to be the deprotonation from position 2 or 3 of D-dopachrome. If this is the case, the imino nitrogen of Pro1 takes part in the deprotonation, in which the proton at position 2 or 3 must be held close to the imino group. Lys32 is located at a favorable position; however, Arg93 is too distant from Pro1. Thus, we speculate that the carboxyl group of D-dopachrome may be located close to Lys32. However, it is still possible that Arg93 is able to contribute to the catalytic mechanism, considering the conservation of this residue in DOPD and CHMI.

Besides the essentiality of the 2-carboxyl group for interaction with the active site, we speculated that two oxygen atoms at positions 5 and 6 of D-dopachrome are involved in the substrate recognition. To investigate this possibility, several chemical reagents were tested. 5-Hydroxyindole-3-acetic acid showed inhibitory activity, but less than indole-3-acetic acid. However, 5-hydroxyindole, 5-hydroxy-L-tryptophan, and serotonin failed to inhibit D-dopachrome tautomerase activity of MIF. These results suggested that the oxygen at position 5 was not critical for active site recognition. Likewise, the oxygen at position 6 might not be involved in the binding, because DHICA was a less potent inhibitor than 5-hydroxyindole-2-carboxylic acid.

We found that 2,3-indolinedione was the most potent inhibitor of MIF among the reagents tested. 2,3-Indoline-

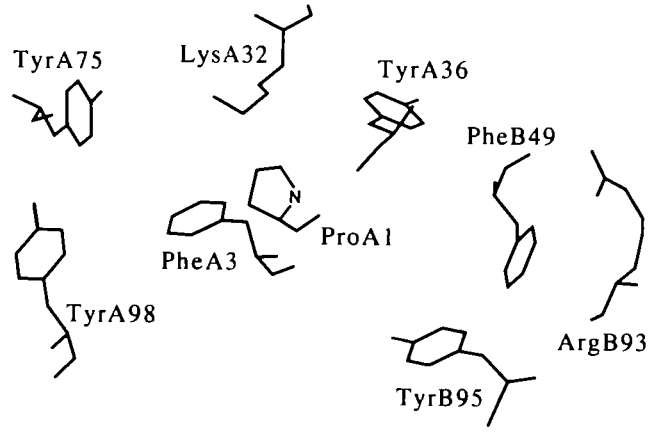


Fig. 3. **Possible catalytic pocket of rat MIF.** The side chains of aromatic residues and two potential carboxyl-binding residues are shown. The distance from the imino nitrogen of ProA1 to the ε-nitrogen of LysA32 is 7.2 Å. The distance from the imino nitrogen to the side chain nitrogen of ArgB93, which is closer to ProA1, is 13.3 Å.

dione is known to act as an inhibitor of several enzymes in a competitive, noncompetitive or uncompetitive fashion (*23, 24*). In certain cases, 2,3-indolinedione inactivated enzyme activities irreversibly by reacting with nucleophilic residues. We speculate that the inhibition of MIF by 2,3-indolinedione is primarily competitive, because this compound shares similar structural features with dopachrome and other indole-ring-containing inhibitors. The carbonyl oxygen at position 2 or 3 of 2,3-indolinedione is presumed to act as a substitute for the carboxyl group of D-dopachrome. The strong inhibitory effect of 2,3-indolinedione on the D-dopachrome tautomerase activity of MIF may be due to the unique position of its oxygen.

Finally, we found evidence for the importance of the 2-carboxyl group and hydrogen atom at position 2. To confirm these findings, precise structural analysis is required. In this context, the crystal structure of an inhibitor-enzyme complex will give further insight into the mechanism of substrate recognition. We are currently investigating the structure of MIF-2,3-indolinedione complex by X-ray crystallographic analysis.

We are grateful to T. Kuriyama and M. Fujinaga for their technical assistance.

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