Structure and Function of *Salmonella typhimurium* Orotate Phosphoribosyltransferase: Protein Complementation Reveals Shared Active Sites†

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ABSTRACT: A solvent-exposed loop, comprising residues 98−119 of *S. typhimurium* orotate phosphoribosyltransferase (OPRTase), is at the subunit interface of the dimeric enzyme, and its amino acid side chains potentially contact active sites on either subunit. A portion of the loop (103−107) appears to be mobile on the basis of the X-ray structures of enzyme•OMP [Scapin, G., Grubmeyer, C., & Sacchettini, J. C. (1994) *Biochemistry 33*, 1287−1294] and enzyme•PRPP•orotate complexes [Scapin, G., Ozturk, D. H., Grubmeyer, C., & Sacchettini, J. C. (1995) *Biochemistry 34*, 10744−10754]. Lys-103, which is essential for activity [Ozturk, D. H., Dorfman, R. H., Scapin, G., Sacchettini, J. C., & Grubmeyer, C. (1995) *Biochemistry 34*, 10755−10763], may thus be functional in the active site formed by the adjacent subunit. Asp-125 is an essential residue that is in the middle of the active site. Equimolar mixtures of the nearly inactive K103A and D125N mutant ORPTase subunits produced approximately 21−23% of the enzymatic activity of the wild-type OPRTase. Heterodimer formation in the complemented mixtures was evidenced by various physical methods. Thus, the active site of OPRTase requires Asp-125 from one subunit and Lys-103 from the adjacent subunit. As predicted from the three-dimensional structure, increased activity resulting from complementation was also observed with mixtures of the K103A mutant and the poorly active K73A and K73Q mutants but not with mixtures of D125N and either K73A or K73Q mutants. Neither K103A nor D125N mutants exhibited negative complementation with the wild-type enzyme. A K103A/D125N double mutant enzyme was also constructed and was able to inactivate wild-type enzyme. Negative complementation between the wild-type and double mutant enzyme was used to determine the subunit dissociation rate for the wild-type dimer, $k_D = 0.013$ min^{-1}.

Many enzymes have mobile loops that occlude the active site during catalysis. Well-documented examples are HIV-1 protease and triose phosphate isomerase (TIM). HIV-1 protease dimer forms a single active site whose occupancy by inhibitors causes 10-residue "flaps" to move up to 7 Å to cover the active site (Wlodawer & Erickson, 1993). In TIM, a loop of 10 residues moves as a rigid body to shield the active site during catalysis (Lolis & Petsko, 1990). Extensive mutagenesis studies of triose phosphate isomerase have suggested that the function of the loop is to restrict access of solvent molecules to the active site, preventing an alternative reaction of the enediol phosphate intermediate and allowing productive catalysis (Pompliano et al., 1990). X-ray structural analysis frequently fails to reveal significant electron density for mobile peptide loops. *Escherichia coli* B glutathione synthetase provides an example of this type of behavior. A 17-residue loop is not visualized in either unliganded or ATP-containing enzyme forms (Tanaka et al., 1992; Yamaguchi et al., 1993), although mutagenesis has demonstrated that the loop is essential for catalysis (Tanaka et al., 1993).

The structure of orotate phosphoribosyltransferase, which transfers the ribose 5-phosphate group from PRPP to orotate in *de novo* pyrimidine synthesis, has been solved to high resolution as its enzyme•OMP and enzyme•PRPP•orotate complexes (Scapin et al., 1994, 1995). The position of the reactive C1 of ribose moves by 7 Å between the PRPP and OMP substrates. The enzyme structure reveals a relatively solvent-exposed active site. The glycosyl-transfer reaction would be expected to proceed via a highly reactive oxocarbonium ion intermediate or an oxocarbonium-like transition state. The instability of such intermediates toward solvent suggests that shielding would be required.

One possible way that shielding could be accomplished is through the movement of a loop comprising residues 98−119, which lies near the subunit interface of the dimer and approaches both active sites. Part of this region (103−107) is disordered in both of the complexes solved to date. However, as shown below, the sequence is highly conserved among OPRTases. Lys-103, which has been shown to be essential for catalysis (Ozturk et al., 1995), is at the border of the disordered region. In this paper, we show that the role of Lys-103 in catalysis is exercised in the adjacent (symmetry-related) subunit of the dimer.

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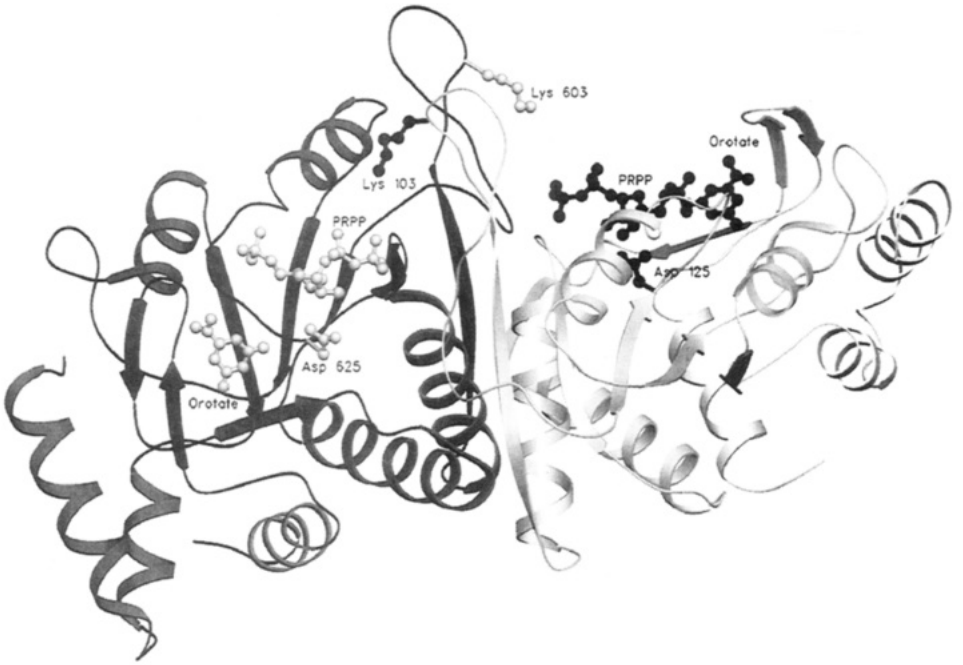

FIGURE 1: Ribbon diagram of the dimeric OPRTase•PRPP•orotate complex. The flexible loop region (residues 98−119) is located at the top of the diagram. The side-chain locations of Lys-103 and Lys-603 (Lys-103 from the adjacent subunit) and Asp-125 and Asp-625 (Asp-125 from the adjacent subunit) are also shown.

EXPERIMENTAL PROCEDURES

Materials. Trizma base, OMP, orotic acid, sodium sulfate, and Luria broth were purchased from Sigma. Restriction endonucleases and ultra-pure grade DTT were obtained from U.S. Biochemicals. DNA preparation and purification kits were from Promega Inc. All other chemicals used were analytical grade. The wild-type *Salmonella typhimurium* OPRTase was isolated from the strain MB13 (Bhatia et al., 1990; Grubmeyer et al., 1993). Mutant enzymes were prepared and purified from the strains listed in Table 1 of the previous paper.

Construction and Purification of OPRTase Mutants. The construction of the lysine mutant enzymes is described in the preceding paper (Ozturk et al., 1995). The D125N mutant enzyme was prepared by the same methods, and its construction and properties will be described elsewhere. Preliminary characterizations of the D125N mutant OPRTase have been reported (Bhatia, 1991; Dessen de Souza e Silva, 1993). The double mutant D125N/K103A was constructed from the plasmids pDOK103A and pDOD125N (Ozturk et al., 1995, Table 1). These plasmids were purified from overnight cultures of strains DOK103A and DOD125N [recombinant plasmid pSP72 carrying the *pyrE* gene with D125N mutation in host BL21(DE3)] using Magic mini-prep kit (Promega Inc.). Each plasmid was first digested individually with endonuclease *Pst*I at 37 °C for 1 h, followed by digestion with *Bss*HII at 50 °C for an additional 1 h. *Pst*I/*Bss*HII digestion yielded 0.46 and 2.89 kb fragments which were purified from 1% agarose gel by Geneclean (Bio 101 Laboratories). The 0.46 kb fragment from the pDOD125N digest (containing the D125N mutation) was ligated with the 2.89 kb fragment from the pDOK103A digest (containing the K103A mutation). The recombinant plasmid (pDODK-1203) was then used to transform BL21(DE3), and the resulting strain was designated DODK1203. Procedures for enzyme expression and isolation have been detailed in the previous paper.

Enzyme Assay. Enzymatic activity in the direction of OMP pyrophosphorolysis was measured spectrophotometrically and quantitated following the procedures outlined in the preceding paper (Ozturk et al., 1995). A unit of activity is defined as that amount of enzyme catalyzing the conversion of 1 μmol of OMP to orotate in 1 min. For the experiments reported here, wild-type, mutant, and complemented OPRTases were prepared in "incubation buffer" which consisted of 100 mM sodium Tricine, pH 8.5, containing 1 mM DTT. We have found that OPRTase solutions, including complemented pairs, kept in this buffer retained more than 90% activity for 1 week at 30 °C.

Complementation Studies with the Wild-Type and Mutant OPRTases. Mutant OPRTases were mixed in pairs in incubation buffer. For each pair, equal amounts of protein (0.5 mg) were incubated in a total volume of 0.4 mL, and 5 μg portions were assayed for enzymatic activity after incubation at 30 °C for the indicated time. Subunit titration and negative complementation experiments were performed in 0.1 mL of incubation buffer, containing a predetermined amount (generally 10 μg) of the subunit being titrated and 2−400 μg quantities of the titrant subunit. Enzymatic activity was measured after 2 h on a 1−5 μg sample. Values of K_M and k_{cat} for complemented pairs were determined as described in the preceding paper (Ozturk et al., 1995).

Detection of Heterodimer. The presence of the heterodimer in the complemented mixtures was demonstrated by nondenaturing 15% PAGE using the system described in the preceding paper (Ozturk et al., 1995).

Alternatively, complemented mixtures were chromatographed on a 0.5 × 5 cm Mono-Q ion-exchange column (Pharmacia LKB Biotechnology, Inc.). Prior to electrophoresis or chromatography, enzymes were desalted according to the procedure of Penefsky (1979), followed by mixing 0.4 mg samples in pairs (total volume 0.8 mL). Following a 2 h incubation period of 30 °C, samples were applied to the column. For elution, a gradient between the starting

buffer (50 mM Tris-HCl, pH 8.0, 2 mM EDTA, and 1 mM DTT) and a limit buffer (starting buffer containing 0.3 M sodium sulfate) was employed at a flow rate of 0.50 mL/min. After sample loading (2 min), a linear gradient was applied from 0% to 25% limit buffer over 10 min, followed by a 20 min gradient from 25% to 45% limit buffer. Enzyme eluted at 27% limit buffer.

RESULTS

The active sites of the OPRTase dimer (Figure 1) are formed as broad, solvent-exposed clefts in the surface of the enzyme and are lined with ionizable residues. The dimer surface exposes two active sites along one face, with both active sites close to the subunit interface. At the subunit interface and exposed on this face are the side chains of residues 98−119 and their symmetry mates from the adjacent subunit. This solvent-exposed sequence extends from β-strand B-2, into a loop (Asn-98 to Arg-119) that connects to β-strand B-3 (Scapin et al., 1994). Side chains of the two solvent-exposed sequences can potentially reach into either of the two active sites. To clarify roles for residues referred to here, we use the term "sequence-related" when a residue interacts with the active site formed by its parent polypeptide, and "symmetry-related" in referring to interactions of a residue with the active site formed by the other polypeptide of the dimer. The X-ray structures of the enzyme indicate that, in the loop region, Arg-99 extends into the symmetry-related active site and contacts the β-phosphoryl of PRPP, whereas the side chain of Lys-100 is active in its sequence-related active site. Past Lys-100, it is difficult to resolve side chains, and for residues 103−107, even the backbone is not well resolved in the two known structures.

Residue conservation in the loop is surprisingly high among sequenced OPRTases (Figure 2) and suggests that residues of the loop are important in catalysis or structure. In particular, residues Arg-99, Lys-100, Glu-101, and Lys-103, which are each conserved, can potentially act as acids/bases, metal ligands, or hydrogen bond donors.

To investigate the possibility that loop movement is a necessary component of the OPRTase reaction, we tested whether nearly inactive K103A OPRTase could complement other mutants. In the center of the OPRTase active site are the conserved aspartate residues of the proposed PRPP binding motif (Hershey & Taylor, 1986; Hove-Jensen et al., 1986). In the PRPP•orotate complex of OPRTase, these aspartates (Asp-124 and Asp-125) are close to the bound PRPP (Scapin et al., 1995), and it is known that their mutation to asparagine results in a substantial loss of enzymatic activity (Bhatia, 1991; D. H. Ozturk, unpublished studies). The structures of substrate complexes of HG-PRTase (Eads et al., 1994) and glutamine-amido-PRTase (Smith et al., 1994) also have these acidic residues located at analogous positions within the active site.

Purified K103A and D125N mutant OPRTases were mixed in buffer and assayed at various times. Figure 3A shows that substantial activity was observed in mixtures of the two nearly inactive mutant enzymes. The maximal enzymatic activity level with the complementing mixture was about 22% of the specific activity of wild type. Attainment of maximal activity required about 2 h, and the final activity was maintained for up to 1 week (Experimental Procedures). The wild-type enzyme treated under the same conditions also retained more than 98% of its original activity. Since neither mutant enzyme displayed significant activity by itself, the observed enzymatic activity in the mixtures must reflect a subunit exchange resulting in active heterodimers.

Sequence	Organism
^{98}NRKEAKDHGEGGN^{110}	*Salmonnella typhimurium*
DRKEAKDHGEGGN	*Sordaria macrospora*
DRKEAKDHGEGGN	*Colletotrichum graminicola*
DRKEAKDHGEGGN	*Trichoderma reesei*
DRKEAKDHGEGGN	*Podospora anserina*
DRKEAKDHGEGGN	*Escherchia coli*
DRKEKKDHGEGGT	*Cryptoccoccus neoformans*
DRKEAKDHGEGGI	*Saccharomyces cerevisiae*
RRKETKDTGTKRL	*Homo sapiens*
RRKEAKAYGTKQL	*Dictyostelium discoideum*
RRKEAKAYGTKKL	*Drosophila melanogaster*
RRKEKKDYGTKRL	*Bos taurus*
CRKERKQYGTGNM	*Naegleria gruberi*
VRKQPKGHGRNAQ	*Rhizobium trifolii*
VRSKPKAHGKGNQ	*Bacillus subtilis*
VRSQAKRHGKGKQ	*Bacillus caldolyticus*

FIGURE 2: Sequence comparison of the highly disordered loop region of *S. typhimurium* OPRTase with other OPRTases. Highly conserved residues are shown in bold including the basic residues Arg-99, Lys-100, and Lys-103. Sequences used were from Scapin et al. (1993) for *S. typhimurium*, le Chevanton and Leblon (1989) for *S. macrospora*, Rasmussen et al. (1992) for *C. graminicola*, Berges and Barreau (1991) for *T. reesei*, Turcq and Begueret (1987) for *P. anserina*, Poulsen et al. (1984) for *E. coli*, Edman and Kwon-Chung (1990) for *C. neoformans*, de Montigny et al. (1989) for *S. cerevisiae*, Suttle et al. (1988) for *H. sapiens*, Boy-Marcotte et al. (1984) for *D. discoideum*, Eisenberg et al. (1990) for *D. melanogaster*, Schoeber et al. (1993) for *B. taurus*, Remillard et al. (1993) for *N. gruberi*, Fennington (1994) for *R. trifolii*, Quinn et al. (1991) for *B. subtilis*, and Ghim et al. (1994) for *B. caldolyticus*.

Titration experiments were carried out to determine the equilibrium between heterodimeric and homodimeric enzyme forms. Figure 3B indicates that, in titrations of K103A with D125N, a sharp break in the plot of observed activity occurred at equimolar amounts of the two subunits. This observation indicated that the complemented heterodimer was strongly preferred in mixtures of the two mutant enzymes.

The individual subunits of the dimer must be able to dissociate into monomers in order to form heterodimers. The equilibrium that results from mixing two different mutants is complex, with five different species present: the two homodimers, the heterodimer, and the two free subunits. The rate of the gain of activity in complementing mixtures is a function of the dissociation rate of the mutant homodimers and the rate of association of the heterologous subunits. The final level of activity observed is governed by the equilibrium between homodimeric and heterodimeric forms and the specific activity of the heterodimer. In experiments with single-site mutants, the rate of approach to maximal activity was first order and was independent of the ratio or the absolute value of subunit concentration. Thus, the rate of subunit dissociation, and not association, is the major contributor to the rate of complementation. A minimal rate of dimer dissociation can thus be estimated by first-order plots of activity vs time at various enzyme concentrations. Such data are shown in the inset of Figure 3A. The single first-order approach to equilibrium observed gave a rate constant of 0.039 min^{-1}.

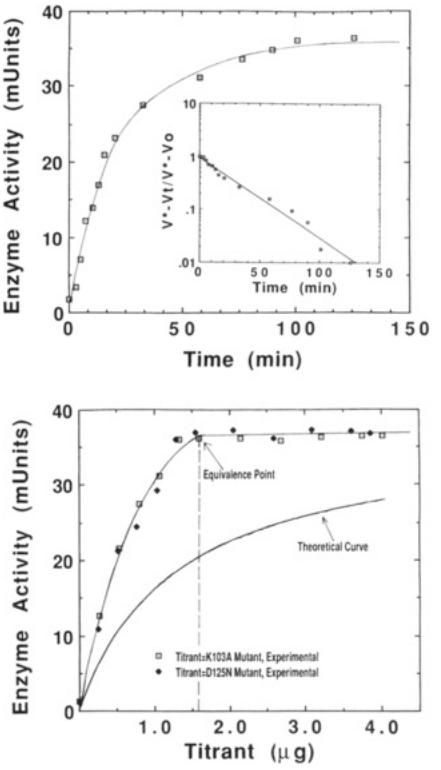

FIGURE 3: Determination of the rate and mode of heterodimer formation. (A, top) Time course of complementation when equimolar amounts of K103A and D125N mutant proteins are mixed at 30 °C. Inset: Semilogarithmic plot of normalized rates of complementation versus time. V^* is the maximum velocity attained, V_t is the velocity at any given time, and V_0 is the velocity at time 0. (B, bottom) Subunit titration of mutant proteins. Each experiment contained about 1.2 μg of the sample mutant protein and the indicated amount of the titrant protein incubated at 30 °C for 2 h. The equivalence point represents equal amounts of the two proteins. The theoretical curve was generated by assuming a random association of mutant subunits. All subunits were assumed to be present in dimeric form, in accord with gel filtration data (Ozturk et al., 1995).

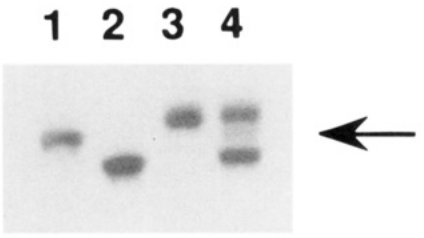

FIGURE 4: Electrophoretic mobility of the wild-type and mutant OPRTases. Nondenaturing 15% PAGE results showing the migration patterns of wild-type OPRTase (lane 1), D125N mutant protein (lane 2), K103A mutant protein (lane 3), and the complemented mixture of K103A and D125N mutant proteins (lane 4). The arrow indicates the position of the heterodimer in the complemented mixture (lane 4).

Physical studies were undertaken to verify that the expected heterodimers did in fact form. In gel filtration, mixtures of mutant subunits were found to behave like the individual homodimeric mutant and wild-type forms of the enzyme, which chromatograph like ovalbumin ($M_r = 45\,000$) and demonstrate a dimeric state. Nondenaturing 15% PAGE showed clear mobility differences between the individual mutant homodimers (Figure 4). When complemented mixtures of subunits were electrophoresed on nondenaturing 15% PAGE gels, a new protein band appeared between that of the two mutants (Figure 4). The band represents the species

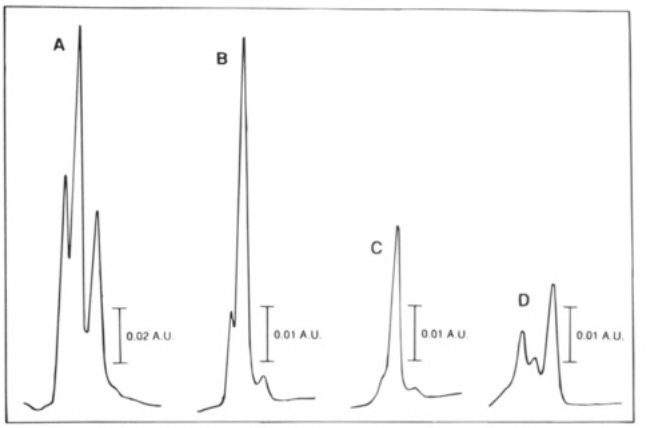

FIGURE 5: Detection of the K103A and D125N heterodimer by anion-exchange chromatography. The elution profiles of the complemented mixtures of K103A and D125N mutant proteins were obtained on a high-resolution Mono-Q anion-exchange column (A). The middle peak from (A) was incubated for 1 h at 30 °C and rechromatographed (B). The middle peak from (B) was incubated for an additional 1 h at 30 °C and rechromatographed (C). The side peaks from (A) were mixed and incubated for 2 h at 30 °C and rechromatographed (D).

of intermediate charge resulting from heterodimer formation. Anion-exchange chromatography on Mono-Q resin clearly resolved three components in mixtures of the mutant enzymes (Figure 5A). Nondenaturing gel electrophoresis of the fractions from the ion-exchange column confirmed that the three chromatographic peaks represented homodimeric D125N followed by the complemented heterodimeric form, with K103A homodimers eluting last (not shown).

Enzyme activity assays carried out on the ion-exchange eluate showed OPRTase activity associated only with peak 2. The specific activity of the heterodimer in this peak was determined to be 29 units/mg. Wild-type OPRTase treated and chromatographed under the same conditions as the complemented mixture exhibited specific activity of 57 units/mg. However, peak 2 still represented only about 55% of the total protein in equimolar mixtures of the two mutant subunits, rather than the 100% expected from the titration experiments. Curves B and C of Figure 5 show that a second and third round of ion-exchange chromatography of material from peak 2 (Figure 5A) continued to resolve three forms but that the heterodimer was now the predominant species. Thus, chromatographic separation does not significantly disrupt the heterodimers. However, when the homodimers of peak 1 and peak 3 from the first round of chromatography were remixed and allowed to complement for 2 h, rechromatography demonstrated only minor amounts of heterodimer (Figure 5D). Thus the mutant enzyme preparation contains some material that fails to complement under these experimental conditions.

Complementation and heterodimer formation were not limited to the K103A and D125N pair. Table 1 shows that K103Q was able to complement D125N, indicating that the complementation was not an artifact of the specific mutation at residue 103. In addition, mutant OPRTases K73A and K73Q also complemented with K103A mutant but not with D125N mutant. These results are expected, since Lys-73 appears from the crystal structures to interact only with substrate bound within the sequence-related active site. Titrations of K103A with K73A or K73Q showed that heterodimers were again preferred. The rate constants for

Table 1: Complementation Studies with Wild-Type and Mutant *S. typhimurium* OPRTase

enzyme pairs tested	final specific activity (% WT)		heterodimer formation[b]
	expected[a]	observed	
K103A + D125N	0.3	22	yes
K103Q + D125N	0.6	20	ND
K103A + K73A	1	41	yes
K103A + K73Q	1	45	ND
K103A + K100A	30	36	yes
D125N + K73A	1	1	NS
D125N + K73Q	1	1	ND
D125N + K100A	30	30	ND
WT + K103A	50	50	yes
WT + D125N	50	50	NS
WT + K19Q	91	91	yes
WT + K100A	80	80	ND

[a] Expected activity was calculated as the sum of units of each mutant divided by the total protein in the mixture and is expressed as the percentage of wild-type specific activity (47 units/mg). [b] As detected by Mono-Q anion-exchange chromatography. ND: not determined. NS: not separable on anion-exchange column.

Table 2: Observed Rate Constants of Complementation between Various Mutants of *S. typhimurium* OPRTase and Effect of Various Ligands on the Rate of Complementation[a]

enzyme pairs	k_{obs} (min^{-1})	$t_{1/2}$ (min)
K103A + K73A	0.010	69
K103A + K73Q	0.011	63
K103A + D125N	0.039	17
+1 mM OMP	0.020	36
+2 mM MgPPi	0.042	17
+1 mM MgPRPP	0.042	17
+1 mM EDTA	0.049	4
+0.55 M urea	0.055	13

[a] Time course experiments were carried out at 30 °C as explained under Experimental Procedures. In the case of added ligands, the ligand concentrations indicated above were included in the incubation mixture. The rate constants were determined from the slope of semilogarithmic plots as shown in the inset of Figure 3A.

complementation between different mutant pairs are shown in Table 2 and ranged from 0.01 to 0.04 min^{-1}. Several ligands were tested for their effect on the rate of complementation (Table 2). 1 mM EDTA, 0.55 M urea, 2 mM $MgPP_i$, and 1 mM MgPRPP were without effect. In the presence of 1 mM OMP the rate of complementation was reduced by approximately 50%.

The kinetics of K103A/D125N, K103A/K73A, and K103A/K73Q complemented mixtures were determined (Table 3). Results indicated a 4-fold increase in K_M for PRPP in the forward direction for the K103A/D125N complemented pair. Kinetic parameters for the other substrates for all complemented pairs were similar to wild-type OPRTase.

Lys-19 is distant from the active site (Scapin et al., 1994), and its mutation to glutamine has little effect on catalysis (Ozturk et al., 1995). The K19Q mutant/wild-type mixture was analyzed by ion-exchange chromatography to determine if heterodimers had in fact formed. Results (not shown) clearly indicated formation of heterodimeric protein, showing that wild-type OPRTase is able to dissociate and reassociate with mutant forms. When mutant K103A or D125N was tested, neither of the mutant subunits had a discernible effect on the activity of wild-type enzyme when mixed under conditions for complementation (Table 1).

Our conclusion that Lys-103 is active in the symmetry-related active site makes the specific prediction that the

Table 3: Kinetic Parameters of Wild-Type and the Active Heterodimers of *S. typhimurium* Orotate Phosphoribosyltransferase for the Forward and Reverse Reactions

(A) Kinetic Parameters for the Forward Reaction

enzyme species	V_{max} (units/mg)	K_M (μM)		k_{cat}/K_M (mM^{-1} s^{-1})	
		orotate	PRPP	orotate	PRPP
WT	88 ± 1	26 ± 1	49 ± 5	1300	690
K103A/D125N	7 ± 0.3	27 ± 1	205 ± 20	99	13
K103A/K73A	33 ± 4	30 ± 0.1	80 ± 0.3	425	160
K103A/K73Q	40 ± 3	25 ± 0.5	52 ± 0.2	600	300

(B) Kinetic Parameters for the Reverse Reaction

enzyme species	V_{max} (units/mg)	K_M (μM)		k_{cat}/K_M (mM^{-1} s^{-1})	
		OMP	PP_i	OMP	PP_i
WT	47 ± 1	5 ± 0.4	33 ± 1	1300	550
K103A/D125N	4 ± 0.1	4 ± 0.3	34 ± 1	380	45
K103A/K73A	21 ± 2	7 ± 0.1	41 ± 0.2	1150	200
K103A/K73Q	26 ± 3	5 ± 0.2	39 ± 0.3	2000	260

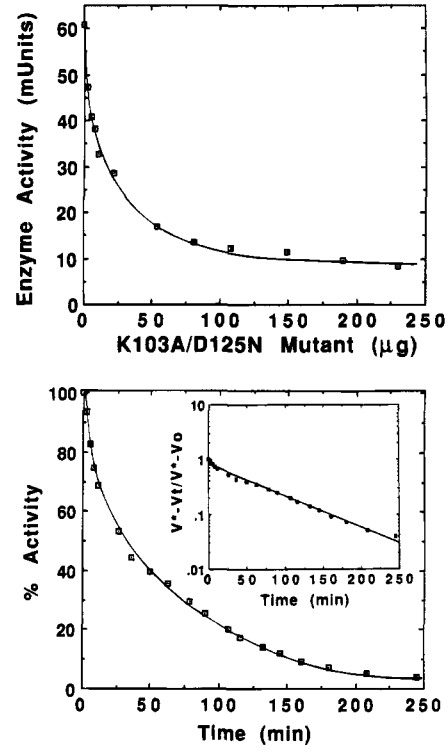

FIGURE 6: Determination of the subunit exchange rate in wild-type OPRTase. Effect of increasing amounts of the double mutant protein K103A/D125N on the activity of wild-type OPRTase (2 μg) in mixtures at 30 °C incubated for 2 h (A, top). Time course of inactivation of wild-type OPRTase (2 μg) with the K103A/D125N double mutant enzyme (100 μg) in a complementing mixture incubated at 30 °C (B, bottom). Inset: Semilogarthmic plot of normalized rates versus time. The definition of the terms V^*, V_t, and V_0 are given in the legend of Figure 3A.

double mutant K103A/D125N would inactivate wild-type OPRTase by heterodimer formation. This double mutant was made and purified. The K103A/D125N mutant enzyme was about 2000-fold less active than wild-type but still behaved as a dimer on gel filtration and migrated like the wild-type enzyme on nondenaturing 15% PAGE gels. Figure 6A shows that, with a 10-fold excess of double mutant enzyme (50 μg) over wild type (5 μg), activity loss was greater than 80%. The rate constant for inactivation of wild type by the double mutant was $k = 0.013$ min^{-1} (Figure 6B, inset). The behavior of the double mutant enzyme provides an important

confirmation of the prediction that OPRTase active sites are shared between the two subunits.

DISCUSSION

The major observation in this paper is that mutation in Lys-103 of *S. typhimurium* orotate phosphoribosyltransferase can be substantially compensated by *in vitro* complementation by the nearly inactive D125N mutant protein or the poorly active K73A or K73Q mutant proteins. Physical evidence (nondenaturing 15% PAGE and chromatography) demonstrated that the increase in activity upon mixing mutant proteins could be correlated with heterodimer formation. The observation clearly demonstrates that OPRTase has active sites that are functionally shared between residues of the two monomers, with Lys-103 exercising its role in the symmetry-related active site (Figure 1). Although mobile loops are known to be present in active sites of several enzymes (Wlodawer & Erickson, 1993; Yamaguchi et al., 1993; Tanaka et al., 1992; Lolis & Petsko, 1990), this observation represents a novel case in which a mobile loop enters the active site from the adjacent subunit. In addition, complementation clearly demonstrates that the active form of OPRTase is the dimer and gives a rate constant for the relatively rapid subunit dissociation of the native enzyme.

Protein-level complementation can result from different mechanisms. In the scaffolding mechanism, proposed by Crick and Orgel (1964), folding of the mutant enzyme is defective, and the adjacent subunit serves as a template to help in the folding process. This explanation is not likely in our case, since mixtures of wild-type and mutant enzymes were not any more active than their separate activities, even though the relevant heterodimers could be shown to form. In addition, the physical tests applied to the mutant OPRTase homodimers (Ozturk et al., 1995) all suggested that they were not substantially deranged, although it is possible that local conformational changes could take place.

Complementation can also occur when active sites are made up of residues from two subunits, and a functional active site can thus be formed from the properly folded components of two defective subunits. The experiments reported here show that Lys-103 of *S. typhimurium* OPRTase contributes to catalysis at the symmetry-related active site. A wealth of structural examples exist which show that active sites are frequently located at subunit interfaces and often appear to involve contributions from both subunits (Wente & Schachman, 1987; Tobias & Kahana, 1993; Frimpong & Rodwell, 1994). A particularly well-explored case is that of aspartate transcarbamoylase, in which complementation by inactive mutants confirmed the results of X-ray diffraction studies (Wente & Schachman, 1987; Eisenstein et al., 1992; Powers et al., 1993).

What is the function of Lys-103 and the loop which contains it? Two classes of possibilities exist. First, it may be that Lys-103 interacts with the pyrophosphoryl moiety of PRPP during the transition state for 5-phosphoribosyl transfer, allowing the latter group to move within the active site during catalysis (Scapin et al., 1995). An alternative proposal is that interactions between K103 and amino acid residues or bound ligands on the symmetry-related subunit serve to hold the mobile loop in a "closed" position during catalysis, protecting the active site from solvent. No evidence yet exists to exclude either of these possibilities.

Three-dimensional structures of two other PRTases have now been reported: hypoxanthine−guanine PRTase (Eads et al., 1994) and glutamine-amido-PRTase (Smith et al., 1994). In both cases the active site is exposed to solvent, although the chemical reaction probably requires solvent exclusion and thus some type of active site closure. In the case of HGPRTase, the sequence at the C-terminal end of the analogous β-strand B-4, containing residues 103−123, may perform this function; however, this region of HGPRTase could only interact with the sequence-related active site (Eads et al., 1994). In glutamine-amido-PRTase the analogous loop extends into the symmetry-related subunit (Smith et al., 1994).

The minimal subunit dissociation rate for the wild-type dimer, 0.013 min^{-1} ($t_{1/2}$ = 53 min), is relatively rapid and implies that dimer dissociation may be significant within cells. Previous analytical ultracentrifugation studies (Dessen de Souza e Silva, 1993) have suggested that monomer may be present in solutions of wild-type OPRTase, although monomer has not been observed in gel-filtration experiments. Recently, Darke et al. (1994) reported a dissociation constant of 0.023 min^{-1} ($t_{1/2}$ = 30 min) for HIV-1 protease dimers, whereas for L-ornithine decarboxylase, the subunit dissociation required for complementation is complete in less than 5 min (Coleman et al., 1994).

An assumption frequently made in complementation studies is that association between mutant subunits is random, allowing simple calculation of concentration of homodimers and heterodimers. With mutant OPRTases, titration experiments showed preference for heterodimer formation between the K103A mutant protein complemented with each of the D125N, K73A, and K73Q mutant proteins, whereas with negative complementation of the wild-type enzyme by the K103A/D125N double mutant, the homodimers were favored (Figure 6A). Although titration experiments showed these preferences clearly, results of ion-exchange chromatography were more consistent with random association, and nondenaturing 15% PAGE suggested strong discrimination against heterodimers. In fact, given that the mutant proteins may be altered in charge and conformation, nonrandom association seems more likely than random behavior and is well documented. In the case of alkaline phosphatase, Fan et al. (1966) found nonrandom association of various mutant forms, and specific activities for the complemented products varied. In histidinol dehydrogenase (Lee & Grubmeyer, 1987) heterodimers were strongly favored in mixtures of complementing subunits because the ability of the complemented heterodimer to bind Mn^{2+} allowed for a thermodynamic pull toward heterodimer formation.

In conclusion, we have demonstrated that Lys-103 plays an unknown essential role in the OPRTase reaction (Ozturk et al., 1995) and exercises its role in the adjacent active site of the dimer. We are continuing to employ crystallographic and biochemical approaches to elucidate the enzymatic role of Lys-103 and the loop which contains it.

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