twinned along (110) boundaries as is typical with this phase. The reason for the formation of defect-free 123 under certain conditions of precursor conversion remains unresolved and is presently under investigation. The observation of superconductivity in defect-free crystals demonstrates, however, that twinning is not a requirement of superconductivity in the $YBa_2Cu_3O_{6+x}$ system. When the powder shown in Fig. 5 and Fig. 6 (top) is further annealed at 950°C in 4 hours in oxygen, the particles, as seen in Fig. 6 (bottom), remain quite small, ranging in size from 500 to 2000 nm. Moreover, there is no evidence of any liquid phase formation. These observations suggest potential advantages with respect to sintered microstructure.

While we are not aware of any previous, documented examples of superconducting 123 powder prepared at temperatures substantially less than 800°C, there have been several reports that superconducting 123 can be made at low temperatures (for example, 600°C) by thin-film techniques (*18, 19*). However, in a typical thin-film synthesis, the temperature quoted is the substrate temperature, which can be considerably lower than the actual temperature at the surface of the growing film. Moreover, the substrate temperatures reported are about equal to or in excess of the orthorhombic to tetragonal phase transformation temperature in the range of pO_2 (0.003 to 0.35 atm) employed (*20*). Thus, a thin-film synthesis may be regarded as a two-step process, first yielding what is most likely nonsuperconducting tetragonal 123, with oxidation to the orthorhombic form occurring during cooling.

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Effects of Buried Ionizable Amino Acids on the Reduction Potential of Recombinant Myoglobin

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The temperature dependences of the reduction potentials ($E^{\circ\prime}$) of wild-type human myoglobin (Mb) and three site-directed mutants have been measured by the use of thin-layer spectroelectrochemistry. Residue Val^{68}, which is in van der Waals contact with the heme in Mb, has been replaced by Glu, Asp, and Asn. The changes in $E^{\circ\prime}$ and the standard entropy ($\Delta S^{\circ\prime}$) and enthalpy ($\Delta H^{\circ\prime}$) of reduction in the mutant proteins were determined relative to values for wild type; the change in $E^{\circ\prime}$ at 25°C was about −200 millivolts for the Glu and Asp mutants, and about −80 millivolts for the Asn mutant. At *p*H 7.0, reduction of Fe(III) to Fe(II) in the Glu and Asp mutants is accompanied by uptake of a proton by the protein. These studies demonstrate that Mb can tolerate substitution of a buried hydrophobic group by potentially charged and polar residues and that such amino acid replacements can lead to subsantial changes in the redox thermodynamics of the protein.

WE REPORT MEASUREMENTS OF the temperature dependences of the reduction potentials of wild-type and three single-site mutants of human myoglobin (Mb). Specifically, we have investigated mutations in which Val^{68} has been changed to the potentially charged residues Glu and Asp, and to Asn, which is uncharged and polar, in order to evaluate the influences of the local electrostatic field on the redox properties of this protein.

These studies were motivated by extensive theoretical (*1, 2*) and experimental (*3, 4*) investigations of the degree to which a protein can influence the redox potential of a prosthetic group or stabilize separated charge after electron transfer. Important factors that affect the reduction potential of a protein include (i) the nature of ligands at the redox center (*3*), (ii) conformational changes associated with reduction, and (iii) electrostatic interactions of the redox center with charged groups both on the surface (*5*) and in the interior of the protein (*6*). Electrostatic interactions are affected by water molecules and ions both in solution and bound at specific sites on the protein and by dipolar and polarizable groups that are present within the protein (*7*). In a recent report, the magnitude of electrostatic interactions between pairs of charged residues was estimated in mutants of subtilisin (*8, 9*). These studies focused on interactions between residues that are accessible to aqueous solvent. However, little is known about the magnitudes of electrostatic interactions between charged groups that are buried within the protein. Since surface charges are well solvated by water, their interactions with the redox center are expected to be smaller than those of buried charges.

In order to assess the magnitude of various contributions to $E^{\circ\prime}$, studies have typically been made of structurally similar proteins and most extensively for the cytochromes (*4*). Such analyses are complicated in that the proteins studied differ at several positions in their primary amino acid sequence. With the advent of site-specific mutagenesis, the effects of individual amino acid changes on redox thermodynamics can be systematically studied. We have chosen Mb because it has been extensively characterized and because of the recent availability of a cDNA clone for human Mb and an efficient method for overproduction in *Escherichia coli* (*10*). Although an x-ray structure of human Mb is not yet available, the residues in the heme pocket are the same as those found in sperm whale Mb, and the optical, ligand binding, nuclear magnetic resonance (NMR), and redox properties of the two proteins are similar (*10*). Residue

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Val[68] (also denoted ValE11) is situated below heme ring I (*11*) on the distal side of the heme pocket and within van der Waals contact of the heme (Fig. 1). This residue is completely inaccessible to external solvent in sperm whale metaquo-Mb (*11, 12*). Metaquo refers to the heme iron in the ferric state with water as the only exogenous ligand. Valine-68 is readily detected in the NMR spectra of carboxy-Mbs (*13*) and appears in wild-type human carboxy-Mb at essentially the same chemical shift as in the sperm whale derivative (*14*).

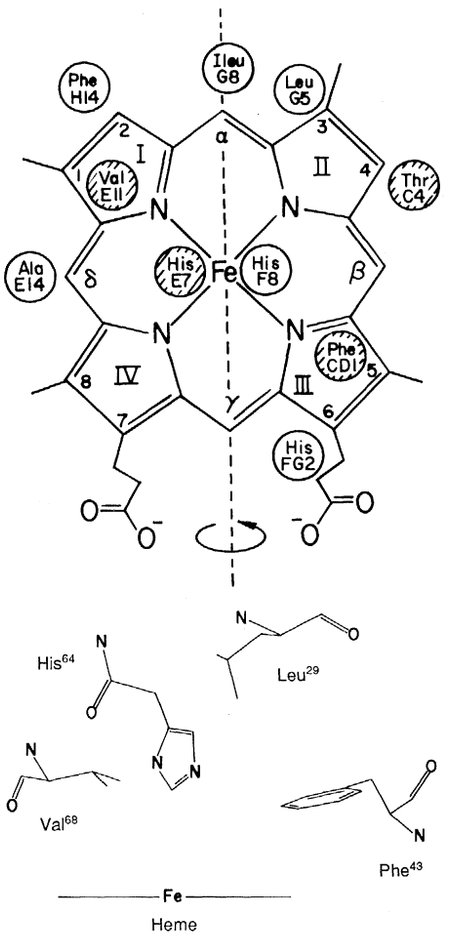

Fig. 1. Positions of amino acids in the heme pocket of sperm whale myoglobin (*26*). Shaded residues on the upper panel and all residues on the lower panel are on the distal side of the heme group.

In the naturally occurring, single-site hemoglobin (Hb) mutants. Hb Milwaukee and Hb Bristol, Val[68] in the β chains is replaced by Glu and Asp, respectively. The Glu in Hb Milwaukee has been shown by x-ray crystallography (*15*) to be weakly coordinated to the heme iron (III), replacing a water molecule that normally occupies this site in wild-type metaquo forms of Hbs and Mbs (*16*). Hb Bristol is much less stable and has not been well characterized (*17*). However, examination of computer graphics models shows that the shortening of the side chain by one methylene group that occurs by replacing Glu by Asp makes it impossible for the Asp carboxyl group to coordinate to iron without substantial protein conformational changes. That Hb can tolerate such drastic changes at this residue position prompted us to replace Val[68] with Glu and Asp in human Mb. These mutants are named V68E and V68D, respectively. To determine whether the Asp is protonated when buried in the protein, we replaced Val[68] by Asn to give the mutant V68N′ (*18*).

All of the mutations were constructed by using the Kunkel method (*19*). In each case the entire protein coding region was sequenced to ensure that no additional mutations had occurred. Expression and purification were essentially as described previously (*10*). The standard free energy, and the entropy and enthalpy changes at *p*H 7.0 associated with reduction of the ferriheme were determined by the use of isothermal variable temperature spectroelectrochemistry (Fig. 2 and Table 1) (*20, 21*). Replacement of Val[68] by the potentially charged residues Glu and Asp decreases $E^{\circ\prime}$ by about 200 mV, whereas replacement by the polar but uncharged residue Asn lowers $E^{\circ\prime}$ by 82.7 mV. These are substantial effects. Also, a subtle interplay occurs between the entropic and enthalpic contributions to changes in $E^{\circ\prime}$.

The mutants have been characterized extensively (*14*). The following are salient features that are relevant for the interpretation of the results in Table 1. Two-dimensional NMR spectroscopy of the CO derivatives of C110A and other mutant proteins demonstrates that there are no major conformational differences between any of these proteins. Electronic absorption spectra are virtually identical (shifts in peak maxima relative to wild type of 2 nm or less) for wild-type and mutant proteins for several different liganded forms, as well as for the reduced deoxy form. A significant exception is the metaquo form of the protein (*22*). The absorption bands at 635 and 1025 nm in the wild-type protein [assigned as ligand-to-metal charge-transfer bands (*22*)] are shifted to 622 and 950 nm in V68D and to 620 and 900 nm in V68E, whereas V68N′ has an absorption spectrum similar to the wild type. Since the interactions of a protonated Asp group with the heme should be similar to those of an Asn, this observation is consistent with the suggestion that the carboxylate side chain at residue 68 in metaquo-V68D is ionized even when buried in the protein. The observed spectral shifts in metaquo-V68E and metaquo-V68D could be due to global conformational changes in these proteins, localized changes in bonding at the heme iron, or purely electrostatic effects of introducing a negative charge near the heme iron. The absence of differences in other regions of the electronic absorption

Table 1. Thermodynamic parameters for the reduction of sperm whale myoglobin (*20*), and for wild-type human myoglobin and four of its site-specific mutants.* $\Delta S^{\circ\prime}$, the total entropy change for the whole-cell reaction (referenced to the standard hydrogen electrode) is given by $\Delta S^{\circ\prime} = \Delta S^{\circ\prime}_{rc} - 15.6$ eu (*25*). $\Delta H^{\circ\prime} = -FE^{\circ\prime} + 298\Delta S^{\circ\prime}$ where F is Faraday's constant. Values of $\Delta H^{\circ\prime}$ and $\Delta S^{\circ\prime}_{rc}$ for Mb are −12.2 kcal/mol and −20.9 eu, respectively.

Myoglobin	$E^{\circ\prime}$ (25°C) (mV)	$\Delta\Delta G^{\circ\prime}$ (25°C) (kcal/mol)	$\Delta\Delta H^{\circ\prime}$ (kcal/mol)	$298\Delta\Delta S^{\circ\prime}$ (kcal/mol)	$\Delta\Delta S^{\circ\prime}_{rc}$ (eu)
Sperm whale	58.8	−0.0	−0.80	−0.80	−2.7
Human	58.9				
C110A	57.0	0.05	−0.04	−0.09	−0.3
V68E	−136.8	4.52	−2.63	−7.15	−24.0
V68D	−132.1	4.41	0.69	−3.72	−12.5
V68N′	−23.8	1.91	2.51	0.60	2.0

*The experimental errors in $E^{\circ\prime}$ and $\Delta S^{\circ\prime}_{rc}$ are ±3 mV and ±1.5 eu, respectively, and were estimated as described in (*20*).

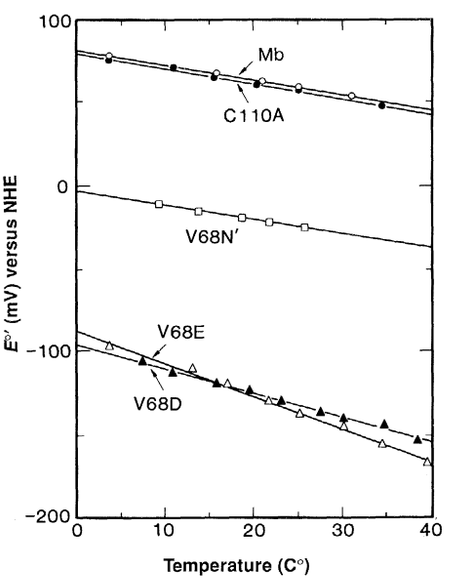

Fig. 2. Redox potential at *p*H 7.0 ($E^{\circ\prime}$) versus temperature for Mb, C110A, V68N′, V68D, and V68E. The entropy change occurring upon reduction in the protein-containing half cell, $\Delta S^{\circ\prime}_{rc} = F(\delta E^{\circ\prime}/\delta T)_P$, is obtained from the slope of these plots.

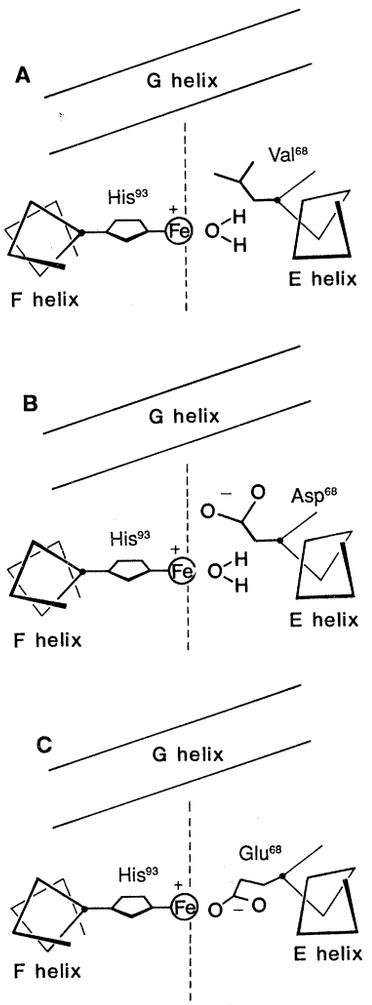

Fig. 3. Schematic representation of the proposed coordination of the heme iron in the metaquo derivatives of (**A**) Mb, (**B**) V68D, and (**C**) V68E.

spectrum, as well as the resistance of both wild-type and mutant metaquo forms of the protein to tryptic digestion, make it unlikely that there are large conformational changes occurring in any of the mutant proteins. By analogy with Hb Milwaukee, in which the Glu replaces the water molecule that is coordinated to the heme iron (III) in the wild-type protein (*15*), it is likely that this substitution occurs in metaquo-V68E. Since Asp cannot coordinate to the heme without a substantial change in protein conformation, these results imply that the observed spectral shifts in metaquo-V68D are due to electrostatic interactions. As expected, ligand-to-metal charge-transfer transitions are highly sensitive to electrostatic effects, whereas the heme-centered $\pi\pi^*$ transitions are little affected. The proposed coordination of the heme iron (Fig. 3) in the metaquo derivatives of Mb, V68E, and V68D is also consistent with results of NMR dispersion studies of these proteins (*23*).

Isoelectric focusing gels indicate that the metaquo forms of V68E and V68D mutants have an isoelectric point (*p*I) that is about 0.5 *p*H units lower than those of the metaquo forms of either wild type or V68N′ and that is similar to the *p*I of reduced (ferrous) Mb, confirming that Glu and Asp are ionized in the metaquo forms of V68E and V68D, respectively. Since the reduced derivatives of V68D and V68E are rapidly oxidized by atmospheric oxygen back to the ferric form, it was not possible to measure the isoelectric point of the reduced forms of these proteins. Myoglobin reconstituted with Zn protoporphyrin IX (ZnPPIX-Mb) has been used as a model for the reduced deoxy state. Wild type and all of the mutant proteins reconstituted with ZnPPIX were found to have approximately the same isoelectric point as oxy-MB, metaquo-V68D, and metaquo-V68E. This suggests that either the carboxylate at residue 68 or a nearby residue (for example, the distal HisE7, Fig. 1), is protonated in ZnPPIX-V68E and ZnPPIX-V68D. This result in turn suggests that reduction of the heme iron in metaquo-V68E and metaquo-V68D is accompanied by uptake of a proton by the protein, which would imply that the reduction potential should increase by approximately 59 mV per unit decrease in *p*H at 25°C (*24*). The $E°(6.0)$ was measured at 25°C for V68D, V68E, and V68N′. The $E°(6.0)$ values for V68D and V68E are greater than $E°'$ values by 64.5 and 53.2 mV, respectively, whereas $E°(6.0)$ for V68N′ is essentially the same as $E°'$, confirming that reduction in the former two proteins is accompanied by proton uptake.

We now consider the origins of the changes in redox thermodynamics summarized in Table 1. Since the redox properties of a protein are determined by many different factors, we restrict our discussion to the observed differences in redox thermodynamics between wild-type and mutant proteins rather than on the absolute values of $\Delta G°'$, $\Delta H°'$, and $\Delta S°'_{rc}$ for each protein. In the metaquo forms of V68E and V68D, the negative charges on Glu and Asp are stabilized by a favorable coulombic, interaction with the heme iron, which has an effective charge of +1. The magnitude of this iron-carboxylate interaction is large because of the close proximity of the charges and because they are surrounded by a nonpolar medium. Upon reduction of the iron, this interaction is lost. The relatively nonpolar protein interior does not appear to be able to solvate an isolated buried negative charge. Hence reduction is accompanied by uptake of a proton.

The $\Delta S°'_{rc}$ value is significantly more negative for both V68E and V68D than for Mb and V68N′. As with the redox potential, this large decrease is a consequence of the *p*H of measurement and the choice of standard state. If all of the observed *p*H dependence of the redox potential in V68E and V68D were due to changes in $\Delta S°_{rc}$, then $\Delta S°_{rc}$ and $\Delta\Delta S°_{rc}$ [$\Delta S°_{rc}$(mutant) − $\Delta S°_{rc}$(Mb)] would increase by about 4.6 entropy unit (eu) per unit decrease in *p*H (*24*). The release of a bound water molecule from the heme iron that occurs upon reduction of metaquo-Mb should be an entropically favorable process. Since the Glu is coordinated to the oxidized heme iron in metaquo-V68E, this process cannot occur. This is likely to be the reason that $\Delta S°'_{rc}$ is more negative for V68E than for V68D. Assuming that the entropy of the water molecule bound to the heme iron is small, the increase in entropy upon transferring this molecule to bulk water is the partial molar entropy of water, equal to 16.72 eu at 25°C. Note that the difference between the $\Delta S°'_{rc}$ values for V68D and V68E is 11.5 eu, which is near this value.

Enthalpy changes upon reduction can be due to either variations in the strengths of specific metal-to-ligand or hydrogen bonds within the protein or to charge rearrangements involving solvent molecules and ions in solution. $\Delta\Delta H°'$ [$\Delta H°'$(mutant) − $\Delta H°'$(MB)] for V68E and V68D are −2.63 and +0.69 kcal/mol, respectively. This difference could be due to the differences in bonding at the redox center in the oxidized states of the two proteins. For V68N′, the +2.51 kcal/mol increase in $\Delta H°'$ is the main contributor to $\Delta E'$. This increase in $\Delta H°'$ is in part due to a favorable hydrogen-bonding interaction involving the Asn, the bound water molecule, and possibly the distal His, which would stabilize the oxidized (metaquo) state of the protein. Such an interaction can be inferred from the observation that the pK_a of the bound water molecule changes from 8.8 in the wild-type protein to 8.1 in the Asn mutant. This corresponds to a free energy change of about 1 kcal/mol at 25°C.

Replacement of Val^{68} by charged and polar residues leads to large changes in the reduction potential of the heme iron. In the metaquo derivatives of V68D and V68E, the negative charge at residue 68 is stabilized by the positively charged heme iron. Upon reduction, the relatively nonpolar protein interior cannot stabilize an isolated buried negative charge, and proton uptake by the protein occurs. Hence the observed changes in redox thermodynamics in these two proteins are strongly *p*H-dependent. These measurements should stimulate theoretical calculations that attempt to reproduce the observed changes in redox thermodynamics.

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24. The half-cell reactions occurring in Mb, V68D, and V68E are

$$Mb^{+}H_2O + e^{-} \rightarrow Mb + H_2O$$
$$V68D^{+}H_2O + H^{+} + e^{-} \rightarrow V68DH^{+} + H_2O$$
$$V68E^{+} + H^{+} + e^{-} \rightarrow V68EH^{+}$$

where e^{-} is an electronic charge. The half-cell reactions in C110A and V68N′ are identical with the one shown for Mb. If we neglect changes in protein structure and charge distribution that occur upon decreasing the *p*H from 7.0 to 6.0, then for the half-cell reactions shown above for V68D and V68E, $E^{\circ\prime} = E^{\circ}(6.0) - 2.303RT/F$. Differentiating with respect to T we obtain $\Delta S^{\circ\prime}_{rc} = \Delta S^{\circ}_{rc}(6.0) - 2.303R$, where R is the gas constant and F is the Faraday constant.
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Evolution of Urea Synthesis in Vertebrates: The Piscine Connection

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Elasmobranch fishes, the coelacanth, estivating lungfish, amphibians, and mammals synthesize urea by the ornithine-urea cycle; by comparison, urea synthetic activity is generally insignificant in teleostean fishes. It is reported here that isolated liver cells of two teleost toadfishes, *Opsanus beta* and *Opsansus tau*, synthesize urea by the ornithine-urea cycle at substantial rates. Because toadfish excrete ammonia, do not use urea as an osmolyte, and have substantial levels of urease in their digestive systems, urea may serve as a transient nitrogen store, forming the basis of a nitrogen conservation shuttle system between liver and gut as in ruminants and hibernators. Toadfish synthesize urea using enzymes and subcellular distributions similar to those of elasmobranchs: glutamine-dependent carbamoyl phosphate synthethase (CPS III) and mitochondrial arginase. In contrast, mammals have CPS I (ammonia-dependent) and cytosolic arginase. Data on CPS and arginases in other fishes, including lungfishes and the coelacanth, support the hypothesis that the ornithine-urea cycle, a monophyletic trait in the vertebrates, underwent two key changes before the evolution of the extant lungfishes: a switch from CPS III to CPS I and replacement of mitochondrial arginase by a cytosolic equivalent.

THE SYNTHESIS OF UREA BY THE ORnithine-urea cycle is widespread among the vetebrates. Marine elasmobranchs (sharks, skates, and rays), the coelacanth, and holocephalan fishes use urea as an important osmolyte (*1*), whereas estivating lungfishes (*2*) and amphibians (*3*) synthesize urea to detoxify ammonia during periods of water stress. Mammals synthesize urea to detoxify ammonia (*4*), a probable prerequisite for living on land, whereas ruminants and some hibernating mammals recycle nitrogen between liver and gut through urea (*5*). Urea cycle enzymes have been detected in only minute activities in some teleostean fishes (*6*) and are absent in others (*7*). Significant urea synthesis has not been reported in teleosts (*8*), and uricolysis or hydrolysis of dietary arginine through arginase are the suggested sources for urea found in teleostean urine (*9*). Components of the cycle (*10*) are identical in all vertebrates, with the exclusion of mitochondrial CPS, which in elasmobranchs is glutamine-dependent (CPS III), and arginase, which is mitochondrial (*11*). In amphibians and mammals, CPS depends on ammonia (CPS I) and a cytosolic arginase liberates urea (*12*).

With few exceptions, teleostean fishes spend most of their lives in water, disposing of waste nitrogen as ammonia with little, if any, metabolic expenditure (*13*). Therefore, Read's early description (*14*) of significant levels of all urea cycle enzymes in the liver of oyster toadfish (*Opsanus tau*), a marine teleost, has remained an anomaly in the literature. Also, the physiological and evolutionary significance of this observation was neglected and the actual occurrence of urea synthesis in *O. tau* was not assessed.

We report that the liver of the related Gulf toadfish, *O. beta*, contains a high titer of all urea cycle enzymes (Table 1) (*15*). Isolated liver cells of *O. beta* and *O. tau* (*16*) rapidly synthesize and release urea (*17*) (Table 2). Rates of urea synthesis in these toadfishes are higher than in elasmobranch hepatocytes and for *O. beta* approach one-third of the mammalian rate (*18*). Otherwise, toadfish liver cells reveal no metabolic peculiarities, with enzyme activities and metabolic flux rates similar to those of other teleosts (Tables 1 and 2) (*19*).

Although toadfish accumulate urea in their plasma (*20*), they are not ureotelic. Like other aquatic teleosts, both toadfish species are ammoniotelic (*21*), whereas excretion of urea is below the limit of detection in *O. beta* and in trace amounts in *O. tau* (*14*). Because the concentrations of urea achieved in the plasma of the toadfish are

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