product was isolated (~70% yield) by silicic acid column chromatography. The radiopurity of VI was established by thin-layer radiochromatography. [^{3}H]-VI, upon oxidation with CrO_3 in 90% acetic acid, gave [^{3}H]-*N*-benzoylglycine (VII) (~40% yield) which was purified by DEAE cellulose acetate column chromatography and showed a single radioactive component upon thin-layer radiochromatographic analysis. Cocrystallization with authentic VII from acetone–hexane showed no significant change in specific radioactivity.[10] [^{3}H]-VI, upon acid hydrolysis,[11] gave [^{3}H]glycine (VIII) which was purified by chromatography on a Dowex-1-acetate column (~98% yield) and whose radiopurity was established by paper and thin-layer radiochromatography.

The stability of the label of [^{3}H]-IV through all of the reactions and procedures utilized in the conversion of [^{3}H]-IV through [^{3}H]-VIII was established in independent experiments.[12]

The [^{3}H]glycine derived from the [^{3}H]ethanolamine 1-phosphate formed enzymatically and [2*RS*-^{3}H]glycine derived from [2*RS*-^{3}H]ethanolamine[12] were each mixed with [1-^{14}C]glycine and incubated with D-amino acid oxidase,[13] an enzyme which, in the catalysis of the conversion of glycine to glyoxylic acid, specifically removes the hydrogen in the *S* configuration at carbon atom 2 of glycine.[14] Glycine and glyoxylic acid (IX) were isolated by Dowex-1-OH column chromatography and paper chromatography. The ratios of ^{3}H/^{14}C in the glycine and glyoxylic acid are presented in Table I.

Table I. Enzymatic Conversion of Glycine to Glyoxylic Acid[a]

Substrate	^{3}H/^{14}C Ratios in glycine and glyoxylic acid recovered after incubation	
	Glycine	Glyoxylic acid
[2-^{3}H,1-^{14}C]Glycine (derived from [^{3}H]-ethanolamine 1-phosphate)		
Experiment 1		0.97
Experiment 2	1.06	0.88
[2*RS*-^{3}H,1-^{14}C]Glycine (derived from [2*RS*-^{3}H]ethanolamine)		
Experiment 1		0.51
Experiment 2 (no enzyme)	0.97	

[a] For ease of comparison, the ratios presented in this table have been calculated in reference to an assigned value of unity in the substrate, *i.e.*, measured ^{3}H/^{14}C ratio in compounds after incubation divided by the measured ^{3}H/^{14}C ratio of the substrate.

The glyoxylic acid derived from the [2*RS*-^{3}H, 1-^{14}C] glycine showed the expected ^{3}H/^{14}C ratio of ~0.5. The glyoxylic acid derived from the labeled glycine (obtained from the [^{3}H]ethanolamine 1-phosphate) showed ratios of 0.88 and 0.97, indicating that the configuration of the labeled hydrogen in the glycine, and hence in the [^{3}H]-ethanolamine 1-phosphate, is *R*. The ^{3}H/^{14}C ratio in the glycine recovered after incubation was essentially unchanged indicating the absence of a significant isotope effect under the conditions studied.

Thus, the enzyme-catalyzed conversion of (2*S*,3*R*)-sphinganine 1-phosphate to ethanolamine 1-phosphate involves the stereospecific incorporation of one atom of solvent hydrogen at carbon atom 2 of the latter compound.

(10) Specific activities: initial, 33.9 ± 0.6 cpm/mg; after one recrystallization, 34.9 ± 0.5 cpm/mg; after two recrystallizations, 32.6 ± 0.3 cpm/mg.

(11) 2 *N* HCl, 100°, 2 hr.

(12) [2*RS*-^{3}H]-IV, Amersham/Searle Co., (292 cpm/μmol) was converted successively, by the reactions outlined above, to [^{3}H]-V (288 cpm/μmol), [^{3}H]-VI (297 cpm/μmol), [^{3}H]-VII (279 cpm/μmol), and [^{3}H]-VIII (288 cpm/μmol).

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T. Akino, T. Shimojo, Y. Miura, G. J. Schroepfer, Jr.*
Departments of Biochemistry and Chemistry
University of Illinois, Urbana, Illinois 61801
and Rice University, Houston, Texas 77001*
Received November 2, 1973

Reactivity Characteristics of Cytochrome *c*(III) Adduced from Its Reduction by Hexaammineruthenium(II) Ion

Sir:

Extensive interest in the redox properties of mammalian cytochrome *c*, a particularly well-studied component of the mitochondrial respiratory system,[1-3] has been buttressed by X-ray determinations of the oxidized and reduced structures of this[4] and a related[5] protein in the crystalline state. Recent kinetic studies indicate that one pathway for the homogeneous reduction of horse heart cytochrome c^{III} by simple reagents in aqueous solution can be governed by a rate-limiting event ($k \sim 30$–60 sec^{-1}) within the protein, which has been plausibly interpreted as involving the opening of the heme crevice and/or substitution on the iron center, followed by a rapid redox conversion.[6] The suggestion has been advanced that the physiological reduction of cytochrome *c* might be related mechanistically to this event.[6b]

While some physiological evidence is consistent with this suggestion,[6b] recent results with improved models are suggestive of a considerably more rapid physiological reduction of cytochrome *c*.[7] It seems possible that the observed rate-limiting event might be accelerated *in vivo*, *e.g.*, through the assistance of heme crevice opening by the reductase. Alternatively, reduction could occur *via* what has been called[6] a "remote" pathway, a description which is apparently intended to include all possible reactions at the periphery of the protein whether they occur by simple outer-sphere, electrostatic complex outer-sphere,[2] or substitution mechanisms. In this communication we report evi-

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dence for a simple outer-sphere reduction of cytochrome c^{III}, which is sufficiently facile to suggest that the high rates of reduction reported under physiological conditions[7] and an outer-sphere pathway are not inconsistent.

The pseudo-first-order consumption of horse heart cytochrome c^{III} (Sigma Type VI, 2–20 μM) by $Ru(NH_3)_6^{2+}$ in large excess was monitored at 550 and 450 nm on a Durrum-Gibson stopped-flow spectrophotometer interfaced to a Varian 620/i computer. At 25.0° and $I = 0.10$ (Tris-HCl) the reaction obeyed the rate law (time in sec)

$$-\frac{d[\text{cyt } c^{III}]}{dt} = \{(3.78 \pm 0.10 \times 10^4) + (7.0 \pm 0.2 \times 10^7)[H^+]\}[Ru(NH_3)_6^{2+}][\text{cyt } c^{III}] \quad (1)$$

over the ranges 0.96 mM < $[Ru(NH_3)_6^{2+}]$ < 7.78 mM and 3.3 < pH < 7.0 (with ~10 mM acetate buffer at the higher acidities). Values of $\Delta H^{\pm} = 2.86$ kcal/mol and $\Delta S^{\pm} = -28.0$ eu for the acid independent path will be discussed elsewhere.

At pH 7.0, where the hydrogen ion path contributes negligibly, plots of k_{obsd} *vs.* $[Ru(NH_3)_6^{2+}]$ were strictly linear up to 7.78 mM where $k_{obsd} = 293$ sec^{-1}.[8] The absence of any hint of rate stauration below this observed rate indicates that any limitation internally imposed by cytochrome c^{III} on its first-order consumption by $Ru(NH_3)_6^{2+}$ at $I = 0.10$ must occur with a considerably higher first-order constant than 300 sec^{-1}, if at all.

These results demonstrate that reaction occurs by a path that evades the rate-limiting event observed[6] with chromous and dithionite reductions. Instead, the nature of $Ru(NH_3)_6^{2+}$ [3] and the positive charges on both reactants virtually ensure an outer-sphere mechanism in which there is no kinetic benefit to be derived from formation of an electrostatically stabilized precursor complex. Thus, our data reflect on the inherent reactivity of cyt c^{III}(aq) toward outer-sphere reduction when there is no kinetic assistance from precursor formation.

An attractive mechanism consistent with our results would involve reaction of $Ru(NH_3)_6^{2+}$ at the peripheral edge of the heme ring, with, perhaps, some penetration into the heme crevice. Except for the absence of electrostatically favorable precursor interactions and environmental variations,[3] this mechanism is similar to one proposed[10] for the physiological reduction of cyt c^{III}.

An alternate proposal for physiological reduction involving a free-radical pathway initiated by the transitory *reduction* of tyrosine-74 has been advanced[4] and questioned[10] elsewhere. Energetic considerations render this mechanism unlikely with our reductant ($E^0 = 0.078$ V, see below). The rather negative para σ_x substituent constant for OH strongly indicates, within current theoretical concepts,[11] that tyrosine is *less* reducible than phenylalanine. Since benzene is not polarographically reducible over the accessible potential range,[11,12] the reduction potential of tyrosine-74 is probably at least as negative as −2 V. Thus, an initial-step activation barrier of $\gtrsim$50 kcal/mol for tyrosine reduction would have to be compensated for by influences which we cannot envisage in order to find agreement with our net $\Delta G^{\pm}$ of 11.3 kcal/mol.

There is no requirement that an *in vitro* redox pathway must correspond to that which is operative physiologically. Nevertheless, when the choice between two outer-sphere mechanistic possibilities for a comparably facile, *in vitro* reaction can be made on grounds as defensible as these, serious consideration must be given to a similar pathway for reductive electron flow within the cytochrome in the two reactions,[13] particularly since the reductase is energetically quite mild ($E^c = 0.22$ V[14]). Important ramifications for understanding physiological behavior would remain even if an alternate physiological pathway is convincingly established since an energetically facile, *in vitro* pathway within cytochrome *c* would then be evaded in some way, perhaps stereochemically, which would demand a physiologically illuminating explanation.

Second-order rate constants comparable to or higher than ours have been reported for cyt c^{III} reduction by energetically esoteric reductants such as the hydrated electron[6,15–17] and reasonably attributed to a remote pathway.[6] However, their high reactivity may be due to much higher driving forces and the mechanisms seem less certain.

That the driving force can contribute to the rate of cyt c^{III} reduction is supported by the apparent applicability of the relative Marcus theory[2,3,18] to our system. We have estimated self-exchange rates of (0.2–1) × 10^3 and 3 × 10^3 M^{-1} sec^{-1} and reduction potentials of 0.26 and 0.078 V for the cytochrome[19,20]

(8) (a) At 9.70 mM $Ru(NH_3)_6^{2+}$, values for k_{obsd} fell irreproducibly below the line in several experiments, presumably the result either of some $Ru(NH_3)_6^{2+}$ decomposition[9] at a concentration of 19.4 mM prior to mixing or of mixing artifacts occasioned by observations close to the mixing dead time. A $k_{obsd} = 360$ sec^{-1} in good agreement with the line was obtained in one experiment. (b) Under conditions approximating Sutin's experiments,[6a] pH 6.10 (20 mM cacodylate) and $I = 1.0$ M (NaCl), where the effect of electrostatic repulsion between the positively charged reactants is diminished, $k_{obsd} = 296$ and 870 ± 30 sec^{-1} were obtained with $[Ru(NH_3)_6^{2+}] = 1.01$ and 3.23 mM, respectively. While these results appear consistent in yielding a second-order constant of ~2.8 × 10^5 M^{-1} sec^{-1}, the higher first-order constant must be viewed with suspicion since we have not eliminated the possibility of contributions from mixing artifacts so close to the instrumental dead time.

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and ruthenium[21] couples, respectively, at 25°, pH 7, and $I = 0.10$. These yield a calculated rate constant for our reaction of (3–6) $\times 10^4$ M^{-1} sec^{-1}, in remarkable agreement with the observed value. It seems likely that our reaction would proceed 20–30 times more slowly were it not for the potential difference.

Presumably outer-sphere reductants even milder than $Ru(NH_3)_6^{2+}$ reduce cyt c^{III} with second-order rate constants[22,23] comparable to ours. The physiological reductant of cyt c^{III}, cyt c_1^{II}, also reacts very rapidly in solution, 3.3×10^6 M^{-1} sec^{-1} at 10°,[24] while being only a slightly stronger reductant than cyt c^{II} [24] and substantially weaker than $Ru(NH_3)_6^{2+}$. Our demonstration that an unambiguously outer-sphere reduction of cyt c^{III} can occur with first-order rate constants approximating those observed physiologically and our mechanistic arguments based on energetics provide support for associating the reactivity of cyt c^{III} with a mechanism involving outer-sphere electron transfer to the exposed edge of the heme ring,[2,3,10] collaborated physiologically with stereospecific electrostatic interactions between proteins[10] (consistent with the temperature dependence of the cyt c_1^{II}–cyt c^{III} rate reported[24] above 10°).

Finally we note the similarity of our acid-dependent term to that observed above pH 4.4 in the Cr(II)–cyt c^{III} reaction.[6a] While this path almost certainly involves reduction of protonated cyt c^{III} in both cases, it is difficult to accept the protonated sites as being the same since we have not observed the complex behavior reported below pH 4.4.[6a] This behavior was rationalized by a scheme in which one of the extraplanar iron ligands is protonated prior to reduction.[6a] Since this is presumably the initial step in the unfolding of the protein,[25] it conceivably might provide an inner-sphere reductant such as Cr(II) with a more facile path than an outer-sphere reductant. A rather speculative alternative for the site of protonation in our case is Thr 78. This idea is based on the suggestion that the physiological reduction of cyt c^{III} might be facilitated on protonation of this residue by a suitably positioned acidic function on the reductase.[10] If, in fact, the reductase protonates a cytochrome residue prior to reduction, it seems feasible that sufficiently acidic solutions could act similarly.

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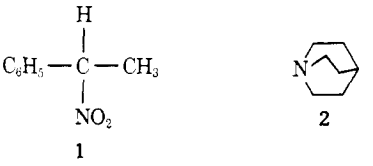
Ralph X. Ewall, Larry E. Bennett*
Department of Chemistry, San Diego State University
San Diego, California 92115
Received October 10, 1973

Unusual Catalytic Activity of Thiophenoxide Ions

Sir:

We have discovered a catalytic effect of thiophenoxide ion pairs which may have considerable importance in helping to understand the mechanisms of certain important enzyme-catalyzed reactions. By way of background we have been studying the racemization of 1-nitro-1-phenylethane (**1**) in propionitrile as catalyzed by



1 2

amines and by mixtures of amines and acids. There are, of course, extensive and important studies by Cram, by Streitwieser, and by others of catalysis by strong bases in a variety of nonaqueous solvents.[1-3] But there have been relatively few studies of catalysis by weak acids or bases in aprotic solvents, and this field certainly merits further work.[1,4-13]

At 25° typical rate constants for catalysis of racemization of **1** are 4.06×10^{-3} for triethylamine, 18.7×10^{-3} for *N*-methylpyrrolidine, and 192×10^{-3} for quinuclidine (**2**), all M^{-1} sec^{-1}. Mixtures of amines with 2,4-dinitrophenol show uv–visible absorption curves similar to those of tetraethylammonium 2,4-dinitrophenoxide and thus they contain hydrogen bonded ion pairs,[14,15] the acid–base reaction being virtually quantitative. Rates of racemization are predictable on two simple hypotheses: (1) with an excess of amine present the 2,4-dinitrophenol is converted quantitatively to ion pairs, and (2) the only catalytic species present is remaining free amine, the dinitrophenoxide ion pairs being catalytically inactive. Separate tests with tetraethylammonium 2,4-dinitrophenoxide showed this also to be inactive.

Contrasting results were obtained with the acid thiophenol, addition of which gave strong rate acceleration. We have not yet obtained a precise value for the equilibrium constant for the reaction between thiophenol and quinuclidine, but the approximate value is 25 M^{-1}. The catalytic constant for the ion pair is then about 2.6

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