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REVIEW

Recent Advances in the Chemistry of an Old Enzyme, Urease

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Urease was the first enzyme to be crystallized and shown to be a protein. Some 50 years after its crystallization, it was shown to be the first example of the nickel metalloenzymes. Whereas urea is degraded by an elimination mechanism in aqueous solution, the enzyme urease catalyzes the hydrolysis of the substrate to carbamate and ammonia as the initial products. This change of mechanism clearly points to the involvement of nickel ion in the chemistry of the enzyme, and this has been independently established. An active-site amino acid residue undergoes a decrease in pK_a' on the binding of the bidentate inhibitor, acylhydoxamate; the mechanism of this acid-strengthening is discussed in detail. The now known list of substrates is large, and special interest attaches to the derivatives of phosphoric acid which are (among others) simultaneously substrates and inhibitors of the enzyme. The mechanism of this inhibition has been elucidated. An active-site peptide from the jack bean enzyme shows very high sequence homology with that from *Klebsiella oxytoca* in accordance with predictions based on the involvement of nickel in the chemistry of the enzyme. A method has been developed to enable the nickel ion at the active site of the enzyme to be replaced by other bivalent metal ions. This relies on the fact that although the enzyme is very stable in 20–50 mM sulfite, the metal ion is slowly lost in very dilute sulfite; the system remains completely soluble, and the electrophoretic and ultracentrifugal properties of the nickel-depleted enzyme are the same as those of the native enzyme.

INTRODUCTION AND DEDICATION

Urease! What a picture the word conjures up for all those who have had even the most fleeting flirtation with chemistry. I first heard the word in 1949 when in my freshman year at the University of Queensland, that great teacher Gilbert Jones (*1*) revealed that *urease* was an absolutely *specific enzyme,* and I added three new words to my vocabulary: I guess that story could be repeated mutatis mutandis many many times. But urease has always had some kind of aura since James Sumner (*2*) first crystallized the enzyme in 1926.

In this article, I shall explore some highlights of our recent work on the enzyme, and in so doing I wish to acknowledge the contribution of Myron Bender to this endeavor, and to dedicate the article to his memory. I had the privilege of working with a very active Dr. Bender from 1959 to 1962, and in my last year with him he

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asked me what systems I might first essay when I became an independent worker. My altogether too quick response was that one of them would surely be urease, because as often discussed with my good friend and fellow worker, Ken Connors, it contradicted everything I thought I knew. Myron enjoined with slight exaggeration that urease had been around for 50 years with little excitement, and probably would be in 50 years' time. I hope this paper demonstrates that his pessimism was not entirely warranted, although there have been many occasions, particularly in the early years of this endeavor, when I surely felt it was.

It is altogether appropriate that I pay tribute to Muriel also. Not only was her love for Myron transparently evident in her every word and action, but she was also exceedingly kind to this lone Australian adrift in a midwestern sea, and helped in so many ways to ease me into the new vernacular.

BACKGROUND

A nickel(II) metalloenzyme. Urease is indeed a venerable enzyme with a history dating securely from 1864 (*3–5*). It was the first enzyme to be crystallized (*2*), and it featured in the controversy over the very nature of enzymes (*6–9*). Sumner had also shown that the enzyme was pure protein devoid of any organic cofactor, and that it contained neither iron nor manganese (*2*). However, in spite of the brilliance of Sumner's experimental technique and the extremely high quality of his observations, when he left the playing field there was no satisfactory description of the chemistry of this enzyme.

The search for a metal ion in urease began early in our own work (*10, 11*) and was a direct consequence of an exhaustive and unsatisfying exploration of protein side chain chemistry as an explanation of the data available at that time. These analyses which were carried out by arc spectrography in the laboratory of Dr. Bert Vallee failed to solve our problem, and indeed impeded progress for some 6 years (*12*). However, there were other developing pointers to metal ion involvement and this led to our subsequent reexamination of the enzyme for metal ions, and to the discovery that the enzyme is a nickeloprotein.

When Walter Mertz examined nutritional trace element research in 1969, nickel was unique among the elements of the first transition series, since all its neighbors [vanadium, chromium, manganese, iron, cobalt, copper (and zinc)] had been reported either to be essential trace elements or to have some particular biological function (*13*). He observed: "This element is particularly interesting chemically because of its easy transition between several coordination structures." However, as noted by Nielsen (*14*), before 1975, any proper examination of nickel deficiency was made exceedingly difficult because of the analytical techniques available at that time, and inadequate control of nickel contamination. It was in this year that we reported that jack bean urease contained stoichiometric nickel and that this represented the first simple biological role for nickel (*15, 16*).

At the same time, it is important to recognize that a specific requirement for nickel had been found 10 years earlier in the growth of *Alcaligenes eutrophus* (*Hydrogenomonas*) when carbon dioxide and hydrogen were the sole sources of

carbon and energy (*17*). Ni(II) could not be replaced by Mn(II), Co(II), Cu(II), or Zn(II), but nickel ion was not required when an organic carbon source (fumarate or succinate) was available to the bacterium (*17*). It was not until 1981, however, that work from Friedrich's laboratory correlated this nickel requirement with the formation of a hydrogenase (*18*), a class of enzymes that uses molecular hydrogen to reduce a variety of electron acceptors (*19–21*). In the following year, the nickel content of the purified enzyme was determined (*22*). In 1981, Graf and Thauer reported that the purified hydrogenase from *Methanobacterium thermoautotrophicum* was also a nickel enzyme (*23*). At about the same time, other work in the laboratories of Thauer (*24*), Wood (*25*), and Wolfe (*26, 27*) provided additional evidence for the involvement of nickel in enzymology.

Since the time of these original observations, the role of nickel in biology has been reviewed (*28, 29*) and many Ni(III) enzymes have been reported in what is a burgeoning area (*30–34*): the excellent recent review of Ankel-Fuchs and Thauer (*29*) provides an interesting and very useful chronology of the main events.

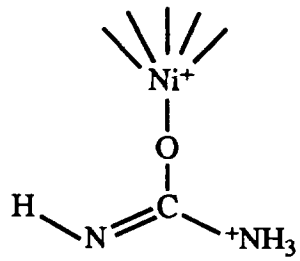
However, urease remains the only well-defined example of a Ni(II) metalloenzyme. Moreover, it is now clear that ureases from a wide variety of sources are nickel-containing enzymes. In all the examples to date, nickel has been found by analysis, a nutritional requirement for nickel has been established, or the presence of the metal ion has been strongly implicated through inhibition of enzymatic activity by hydroxamic acids or phosphorodiamidates (*35*).

A hydrolytic enzyme. The urea molecule is very stable. Between pH 2 and pH 12, the nonenzymatic decomposition of urea in aqueous media is independent of pH and has a half-life of 3.6 years at 38°C (*36–38*). It has been shown that this reaction is an *elimination* whose only products are ammonia and cyanic acid (*39–41*). The rate of the elimination reaction increases above pH 12, presumably owing to the incursion of specific base promotion of the elimination reaction (pK'_a of urea, ~14). Model reactions support this chemistry (*42–44*): one example is the alkaline hydrolysis of *p*-nitrophenyl carbamate which has a k_{-OH} of 2.64×10^5 M^{-1} s^{-1} at 25°C (*45*). Below pH 2, the rate of decomposition of urea *decreases*, apparently because of the protonation of urea (pK'_a of urea·H^+, ~0.2) (*46*). We have reexamined the reaction of [^{13}C,^{18}O]urea in strong acid (to 8.04 M perchloric acid) at 100°C in an attempt to find evidence for the *hydrolysis* of urea in aqueous solution, but while there may be some hydrolysis of the protonated urea, the results of these experiments are inconclusive (*47*).

Urease catalyzes the *hydrolysis* of urea to carbamate and ammonium ion (*11*) as shown in

$$H_2N{-}\overset{\overset{O}{\|}}{C}{-}NH_2 + H_2O \rightarrow H_2N{-}COO^- + {}^+NH_4 \qquad [1]$$

and this fact points to the special chemistry of the enzyme system, since the spontaneous *hydrolysis* of urea has not been observed in aqueous solution. The enzyme converts an elimination to a hydrolysis and this suggests a key role for Ni^{2+} (as a Lewis acid) in the chemistry of urease. Undoubtedly metal ion stabilization of the zwitterion (*40, 41*) as in **1** would operate against elimination and



1

predispose the coordinated urea to attack by water or coordinated hydroxide ion (hydrolysis), to yield carbamate. This is possibly the essence of the chemistry of the enzyme.

Specificity. Woven into the rich tapestry that is enzymology at a very early stage was the concept of absolute specificity, and urease became its textbook paradigm. The 1965 report from Fishbein's laboratory that *N*-hydroxyurea is also a substrate constituted a very significant advance (*48, 49*). The molecule is at once a substrate and inhibitor (owing to the hydroxamic acid function), and its behavior is classically characteristic of a substrate molecule that can form an abortive enzyme–substrate complex (*11*).

Physicochemical properties. The major physicochemical properties of the enzyme are summarized in Table 1. It should be noted that the subunit weight of the polypeptide of 840-amino acid residues is 90,777 based on the corrected amino acid sequence of Mamiya *et al.* (*50–52*). This corresponds to a molecular weight of 545,365 for the hexameric molecule which contains 12 g-atoms of nickel.

Interactions as the active sites of enzymes. Given that all enzymes use what can be most simply, usefully, and generally described as acid–base chemistry, important factors which may influence the chemistry at the active site of any enzyme are: (i) the interaction of a hydrophobic environment with a charged group of any kind, whether it be on a protein side chain, an inhibitor, or a substrate; and (ii) the interaction of charged protein side chains with another protein side chain, inhibitor, or substrate. These interactions have completely predictable effects: a hydrophobic environment will stabilize a neutral acid species, leading to an increased pK'_a, and will destabilize a cationic acid species leading to a decreased pK'_a. A positively charged protein side chain will lower the pK'_a of a nearby neutral or cationic acid, while similar interaction involving a negatively charged protein side chain will increase the pK'_a of the adjacent acid (*56*). It follows that a net increase or decrease of charge at the active site of an enzyme on the binding of an inhibitor or substrate will decrease or increase the pK'_a of an appropriately located acidic group. These effects may be expected to assume significance where the substrate or inhibitor are acidic molecules, and especially where the charge at the active site of the enzyme may undergo *differential alteration,* as may commonly occur in metalloenzymes. The general principles discussed above are unarguably of great diagnostic value (*57, 58*), although there appears to be no previous generalization

TABLE 1

Some Physicochemical Properties of Urease

Property	Value	Ref.
Molecular weight	545,365 (sequence data + Ni^{2+} content)	*(50–52)*
Molecular weight	590,000 ± 30,000 (equilibrium ultracentrifugation)	*(53)*
Equivalent weight	96,600 ± 1000	*(53)*
Polypeptide mol wt	90,777 (sequence data)	*(50–52)*
Subunit mol wt[a]	95,000 ± 5000 (equilibrium ultracentrifugation)	*(53)*
Subunit mol wt[b]	~93,000 (PAG–SDS electrophoresis)	*(53)*
Subunit mol wt[c]	~96,000 (PAG–SDS electrophoresis)	*(47)*
Partial specific volume	0.734 $cm^3\ g^{-1}$ (gravimetric)	*(53)*
	0.738 $cm^3\ g^{-1}$ (amino acid analysis)	*(54)*
Diffusion coefficient ($D_{20,w}$)	$3.27 \pm 0.32 \times 10^{-7}$ $cm^2\ s^{-1}$	*(54)*
Sedimentation coefficient ($s^{\circ}_{20,w}$)	19.35 S	*(54)*
ε_{280} (gross)	62,000 $M^{-1}\ cm^{-1}$ at 280 nm[d]	*(53)*
$A^{1\%}_{1\,cm}$ (net)	6.20 at 280 nm	*(53)*
Specific activity	93 (mkat $liter^{-1}$)/A_{280}	*(15, 53)*
Nickel content	2.00 ± 0.12 ions per subunit	*(55)*

[a] In 0.05 M $TrisH^+Cl^-$ buffer, pH 7.4 (0.15 M in KCl, 1 mM in EDTA, 6.0 M in guanidinium chloride, 0.1 M in 2-mercaptoethanol).

[b] Sample dialyzed into the buffer in footnote *a* for 20 h at 28°C.

[c] Sample preequilibrated at 100°C for 2 min in 0.05 M $TrisH^+Cl^-$ buffer, pH 8.0 [1% (w/v) in SDS, 1% (v/v) in 2-mercaptoethanol].

[d] Based on the 96.6-kDa subunit.

to the substrate itself, and the significance of differential alteration of charge at the active site has gone unheralded.

As an example, consider such *acid strengthening* effects in a model for urease in which a neutral acid and ^-OH (or water) are two of the ligands coordinated to the active-site nickel ion. If a bidentate inhibitor of the enzyme such as an acylhydroxamate ion (X^-) displaces both these functions, not only will the nickel center itself gain an additional positive charge but the active site will also, if the displaced neutral acid ligand (from Asp, Glu, Cys, or *Tyr*) *(59)* becomes fully protonated (Eq. [2]). If instead of ^-OH, water were the coordinated nucleophile, Eq. [3] would describe the event. Therefore, while Eq. [2] could account for the acid strengthening of a residue under the immediate influence of the nickel ion (which increases in positive charge by one unit), it is clear that Eq. [3] cannot. In like manner, Eqs. [4] and [5] describe the parallel systems in which a positively charged acid ligand (from *His* or Lys) is released. Here the charge on nickel either does not alter (Eq. [4]) or decreases by one unit (Eq. [5]).

$$X^- + HO\text{—}Ni\text{—}A\text{—}Enz + 2H^+ \rightarrow X\overset{\cdots}{-}Ni^+ + H\text{—}A\text{—}Enz + H_2O \qquad [2]$$
$$\downarrow\uparrow$$
$$H^+ + {}^-A\text{—}Enz$$

$$X^- + H_2O{\cdot\cdot}Ni^+\text{—}A\text{—}Enz + H^+ \rightarrow X\overset{\cdots}{-}Ni^+ + H\text{—}A\text{—}Enz + H_2O \qquad [3]$$
$$\downarrow\uparrow$$
$$H^+ + {}^-A\text{—}Enz$$

$$X^- + HO—Ni^{+}\cdots:B—Enz + 2H^+ \rightarrow X\cdots Ni^+ + H—{}^+B—Enz + H_2O \quad [4]$$

$$X^- + H_2O\cdot\cdot Ni^{2+}\cdots:B—Enz + H^+ \rightarrow X\cdots Ni^+ + H—{}^+B—Enz + H_2O \quad [5]$$

However, protonation of the displaced positively charged acid ligand will in both cases generate a net increase in positive charge in the *immediate vicinity* of the active site. Formally, of course, Eq. [5] is overall neutral with respect to charge alteration even though the nickel center loses one unit of positive charge. What one will observe experimentally in the event described by Eq. [5] will clearly depend on the exact location of the residue whose acidity is being influenced. It should be realized, of course, that neither ^-OH nor water may be a disposable ligand, and that it is not unlikely that the two ligands may both be amino acid side chains as in Eqs. [6], [7], and [8].

$$X^- + Enz—A—Ni—A'—Enz + 2H^+ \rightarrow X\cdots Ni^+ + \begin{matrix} Enz—A—H \\ \Updownarrow \\ Enz—A^- + H^+ \end{matrix} + \begin{matrix} H—A'—Enz \\ \Updownarrow \\ H^+ + {}^-A'—Enz \end{matrix} \quad [6]$$

$$X^- + Enz—A—Ni^{+}\cdots:B—Enz + H^+ \rightarrow X\cdots Ni^+ + \begin{matrix} Enz—A—H \\ \Updownarrow \\ Enz—A^- + H^+ \end{matrix} + H—{}^+B—Enz \quad [7]$$

$$X^- + Enz—B:\cdots Ni^{2+}\cdots:B'—Enz + 2H^+ \rightarrow X\cdots Ni^+ + Enz—B^+—H + H—{}^+B'—Enz \quad [8]$$

In summary, any one of Eqs. [2], [4], [5], [6], [7], or [8] could describe the essential chemistry of an acid-strengthening effect, but not all with equal likelihood. Only Eqs. [2] and [6] use the nickel center itself, and Eqs. [4], [5], [7], and [8] rely on the protonation of the basic amino acid side chain(s) released. On general chemical grounds alone, therefore, Eqs. [7] and [8] could well be the best candidates, provided that in Eq. [7], Tyr is the neutral acid residue.

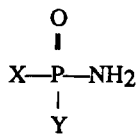
SUBSTRATES

The discovery of a range of substrates for the enzyme has been very significant in delineating a role for nickel ion in urease catalysis (*51, 60*). Secure evidence for the coordination of urea to an active-site nickel ion was later obtained from spectral work (*61, 62*).

The now known substrates for the enzyme are given in Table 2.

The most recent additions are the amides and esters of phosphoric acid with the general structure **2** shown. Phosphoramidate **2a** is an inhibitor of the enzyme because of stoichiometric coordination to the active-site nickel ion (*15, 16, 35, 60, 62*). However, it is now clear that it is also a genuine substrate for urease with a $k_{cat}/K_m = 8 \pm 2\ M^{-1}\ s^{-1}$ ([**2a**] = 8.4 mM) at 38°C.

Phenyl phosphorodiamidate (**2d**, Ar = Ph, PPD), *N*-(3-methyl-2-butenyl)phosphoric triamide [**2e**, Alk = $(H_3C)_2C{=}CH$, MBPT], and phosphoric triamide (**2c**)



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a X = HO Y = HO

b X = HO Y = H_2N

c X = H_2N Y = H_2N

d X = H_2N Y = ArO

e X = H_2N Y = AlkC(O)NH

f X = H_2N Y = ArC(O)NH

each rapidly and stoichiometrically inactivates urease, but the inactive enzyme slowly regains full activity at pH 7 and 38°C. The equivalence of the rate constants for the reactivation of the enzyme inhibited by these three compounds identifies a diamidophosphate–nickel complex (**2b**···Ni^{2+}) as the species responsible for inhibition. A different, significantly more labile complex is formed when the inhibitor is not derived from substrate during the enzymatic reaction. The isomeric diamidophosphate–nickel complexes probably differ in strength by virtue of N- vs O-coordination to the metal ion, and constitute the first such enzymatic data (*11*,

TABLE 2

Substrates for Urease

Substrate	k_{cat}[a] (s^{-1})	K_m (M)	k_{cat} at pH 5.2 / k_{cat} at pH 7.0	$10^4 k_{react}$[b] (s^{-1})
Urea	5870	0.0029	0.6	
Semicarbazide	30	0.060	1.7[c]	
Formamide	92	1.06	2.4	
Acetamide	0.55	0.75		
N-Methylurea	0.075	0.22		
N-Hydroxyurea[d]				
Phenyl phosphorodiamidate				0.36 ± 0.03[e]
N-(3-Methyl-2-butenyl)phosphoric triamide[f]				0.34 ± 0.04[e]
Phosphoric triamide				0.36 ± 0.03[e]
N-Benzoylphosphoric triamide[f]				
Diamidophosphate[g]				7 ± 1[h]
Phosphoramidate[g,i]				8.2 ± 0.5[j]

[a] pH 7.00, 38°C (*45*).

[b] First-order rate constant for the reactivation of the enzyme-inhibitor complex which is rapidly formed in the presence of excess enzyme (*63*).

[c] The lower pH was 5.0.

[d] Refs. (*11*, *48*, *49*).

[e] In oxygen-free *N*-ethylmorpholinium chloride buffer (pH 7.0, 1 mM in EDTA).

[f] *N*-Acylamide was reported as a product (*64*).

[g] 1 : 1 complex.

[h] In oxygen-free *N*-ethylmorpholinium chloride buffer, pH 7.0.

[i] $k_{cat}/K_m = 8 \pm 2\ M^{-1}\ s^{-1}$ ($[S]_o$ = 8.4 mM; 38.0°C).

[j] In oxygen-free *N*-ethylmorpholinium chloride buffer (pH 7.11, 1 mM in EDTA, 5 mM in 2-mercaptoethanol, 0.1 M in KCl).

63). The identity of the rate constants for reactivation of urease inhibited by **2a** and **2b** (Table 2) also strongly suggests that **2b** is itself a substrate.

The above data are of critical importance to those who would seek to inhibit urease on a predictable time scale so as to maximize the benefits of urea as a fertilizer. Currently many hundreds of millions of dollars are wasted annually, especially in rice culture, because of the loss of nitrogen (as ammonia) by the action of soil and bacterial ureases on urea.

DIFFERENTIAL ALTERATION OF CHARGE AT THE ACTIVE SITE

Iodoacetate has almost no effect on urease, whereas iodoacetamide alkylates the enzyme slowly and irreversibly. While not yet unequivocally established, this alkylation presumably involves the active-site cysteine (*50*). If, however, the enzyme is preequilibrated with 0.9 mM benzhydroxamic acid, the rate of this reaction is increased at least fivefold. That this phenomenon is not peculiarly related to alkylation by iodoacetamide is demonstrated by the fact that at pH 7.3, the rate of titration of the essential thiol by DTNB is also enhanced some sixfold in the presence of 1.1 mM benzhydroxamic acid. These facts are readily accounted for if the acidity of the essential cysteine has been increased by about 0.8 pK_a units. Mechanisms for this acid strengthening effect on the binding of a bidentate acylhydroxamate ion have already been discussed. Moreover, these observations assume further significance when one explores the chemistry of the nickel-depleted enzyme.

In the native enzyme, only one of the nickel ions is available for titration by hydroxamic acids. For the purpose of discriminating between the two ions, let us call this nickel ion, $Nickel_1$. The result clearly implies that none of the ligands to $Nickel_2$ is replaceable by the bidentate acylhydroxamate in the native enzyme. We have now succeeded in removing various fractions of nickel from the protein, and as the nickel is removed there is an *increase* in absorbance at wavelengths in the region 350–450 nm. Again, as discussed earlier, this can be explained if the acid strengthened active-site Cys (caused by the removal of $Nickel_1$) competes for $Nickel_2$ ligation. Not only does this provide a straightforward mechanism for the reequilibration of the nickel between the two sites, but it also provides the first experimental *indication* that the two nickel ions may be close together.

ACTIVE-SITE SEQUENCES BY DNA HYBRIDIZATION

In the middle part of this century, a great deal of effort was expended in demonstrating sequence homologies among closely related proteins and enzymes (*65*), and it was discovered that the active-site regions of many related enzymes are very highly conserved. The rise of the techniques of DNA technology and the greatly increased availability of useful structural information from X-ray crystallography have largely changed the face of protein chemistry and made such exercises apparently very much simpler. Nonetheless, problems of synthesis and pro-

duction remain, and this is especially true when comparisons of enzymes from prokaryotic and eukaryotic sources are required.

The complete amino acid sequence of jack bean urease published by Mamiya and his co-workers in 1985 showed that the active-site cysteine (*66*) was residue 592 in the 840-residue peptide (*50–52*). Most of the secure published work on urease refers to the jack bean enzyme, but this system does not readily lend itself to modification studies, and the question logically arises as to just how typical it is of the nickel ureases. We therefore sought a comparison of a *Klebsiella* urease with that from the jack bean, because the prokaryotic systems offer far greater scope for the application of DNA technology.

Using the data available in 1974, Liljas and Rossmann concluded that in metalloproteins of known structure at least two of the metal-binding protein ligands are almost invariably located close together in the polypeptide chain (*67*). Subsequent data strengthen the proposition with examples of separation of two of the protein ligands of generally between one and five residues (*68*).

Kobashi and his co-workers have demonstrated that photooxidation of some histidine residues in sword bean (*69*) urease leads to complete inactivation of the enzyme, together with a diminished ability to bind hydroxamic acids (*70, 71*). It is significant that the only residue affected in these experiments is histidine, and the fact that benzhydroxamic acid protects the enzyme against inactivation resulting from this photooxidation clearly indicates that at least one of the histidine residues affected is at the active site of the enzyme. Thirteen of the 25 histidine residues in the jack bean urease subunit occur between residue 479 and 607, and two of them (His-593, -594) are next to the active-site cysteine residue, Cys-592 (*50–52*). It is now beyond doubt that nickel is essential to the chemistry of the jack bean enzyme. Moreover, if all the nickel ureases experience the same chemistry, because of the demands put on a polypeptide chain appropriately to bind two bivalent nickel ions, one must reasonably expect that the active sites of all these enzymes will be very highly conserved.

As a first step toward establishing the generality of active-site structures in the nickel ureases, it was necessary to check the available data on the sequence of the jack bean enzyme. mRNA was prepared from 18- to 20-day-old jack beans, and several cDNA libraries were prepared in λgt10. Two synthetic oligonucleotides were designed on the basis of Mamiya's sequence of the jack bean enzyme (52). Both nucleotides were used to screen the libraries. cDNA which encoded the entire urease polypeptide was found on two contiguous cDNAs which were designated pJB2300 and pJB400 which were, respectively, 2300 and 400 bp long. Generation of the full-length enzyme–cDNA was achieved with the aid of reverse transcriptase in the synthesis of the second strand. It is worth noting that the use of DNA polymerase resulted in incomplete synthesis. The sequence of the cDNA leads to a primary amino acid sequence which agrees exactly with that reported earlier (*51, 52*). Further, analysis of the cDNA indicates that the jack bean enzyme is expressed as a mature protein (*72*).

The cloning of bacterial urease genes may be achieved by selecting for ureolytic colonies which produce a pink color on Christensen's agar, a medium which contains both urea and the indicator, phenol red (*73*). In this way, clones were

		Ser	Thr	Asn	Pro	Thr	Arg	Pro	Leu	Thr	Ser	Asn	Thr	Ile	Glu
Jack Bean:	570	TCA	ACA	AAC	CCC	ACA	CGC	CCT	TTA	ACA	TCT	AAT	ACT	ATA	GAC
***K. oxytoca*:**		TCG	ACC	AAC	CCG	ACG	CTG	CCC	TAT	ACC	GTC	AAC	ACC	ATA	GAC
							Leu		Tyr		Val				

		Glu	His	Leu	Asp	Met	Leu	Met	Val	Cys	His	His	Leu	Asp
	584	GAC	CAT	CTT	GAC	ATG	TTG	ATG	GTT	TGC	CAT	CAT	CTG	GAT
		GAA	CAT	CTG	GAT	ATG	CTG	ATG	GTT	TGC	CAT	CAT	CTC	GA

FIG. 1. DNA and inferred amino acid sequences of a highly conserved peptide in the ureases from the jack bean and *K. Oxytoca*. Cys-592 is the active-site thiol in the jack bean enzyme. Base differences are set in boldface.

obtained from *Klebsiella oxytoca* (*73*) and *Bacillus pasteurii* (*74*) by the use of genomic libraries prepared in *Escherichia coli*. The DNA fragments of *B. pasteurii* and *K. oxytoca* which express urease activity in *E. coli* have ~12 and ~10 kbp, respectively. Soluble extracts of the strains of *E. coli* which carried the bacterial enzymes contained proteins (~60 kDa from *B. pasteurii*, and ~68 kDa from *K. oxytoca*) which reacted with antibodies raised against the pure jack bean urease. It should be noted that the nickel-containing urease from *K. aerogenes* has been reported to have an $\alpha_2\beta_4\gamma_4$ structure with approximate M_rs of 72,000 (α), 11,000 (β), and 9000 (γ), where the α-subunit contains two nickel ions (*75*).

We have used jack bean urease cDNA as a hybridization probe at high stringency to identify the most highly conserved region of the *K. oxytoca* urease gene, and we have in fact located a region corresponding to a peptide whose inferred sequence is highly homologous with the sequence of the Cys-592 active-site sequence in jack bean urease (Fig. 1) (*76*). These data provide strong support for the prediction that the active site of urease will be essentially the same regardless of phylogenetic source. The fragmentary data on codon usage by *K. oxytoca* in this sequence show similar bias to the usage by *E. coli* and support earlier analyses of prokaryotic systems (*77, 78*).

The basic idea which was exploited in this study is straightforward: highly conserved regions of proteins or enzymes may be very rapidly scanned by the hybridization at high stringency of the full-length cDNA of one form of the protein or enzyme to subclones of the DNA encoding the other. A central thesis of the work is that the most demanding spatial arrangements of amino acid residues in enzymes will occur at their active sites because of the need correctly to locate binding and catalytic functionalities. This is expected to be especially true of metalloenzymes. To my knowledge, this is the first such application of a simple principle which could obviously be extended to other enzymatic and protein systems with great benefit and the saving of a deal of time.

There may be a valid argument that not all components of the active site will be found using the present procedure, because individual catalytic components are not uncommonly widespread in the primary amino acid sequence. Whether the procedure will always yield a region of the active site is not yet known, but we plan to test its predictive validity on a much wider scale with both pure protein enzymes as well as metalloenzymes. Nevertheless, it is clear that the procedure has much to commend it where a great deal of extraneous mechanistic information

exists. Moreover, by gradually reducing the hybridization stringency, it should be possible to obtain clones which encode other important regions of the enzyme. Figure 1 also reveals that the three histidines in this sequence (residues 585, 593, and 594 of the jack bean enzyme) are exactly conserved, and it is not unreasonable to suggest that at least two of them are likely nickel ligands.

Finally, in this section, it should also be noted that Mobley and Hausinger have reported fragmentary information which demonstrates that the N-terminal sequences of the large subunits of *K. aerogenes* and *Proteus mirabilis* are highly conserved, and that the sequence of residues 4–19 in the *P. mirabilis* subunit shows significant homology with that of residues 274–290 in the jack bean urease polypeptide (*79*).

REMOVAL AND REPLACEMENT OF NICKEL ION

Highly purified urease contains 2 g-atoms of nickel per subunit. At the same time, it should be noted that the enzyme contains negligible Ca, Mn, Fe, and Co (*80*). The nickel is tightly bound: EDTA does not promote the loss of nickel at neutral pH, but the enzyme very slowly loses nickel. Thus EDTA can be used during the purification of the enzyme to protect the protein from adventitious metal ions. However, at low pH (3.6–4.0), EDTA does promote both the removal of the nickel and the irreversible inactivation of the enzyme (*80*). Equilibrium protonation of one or more basic amino acid ligands would account for these data and once again they are consistent with the ligation of a histidine side chain to an active-site nickel. For many years, attempts reversibly to remove and replace the

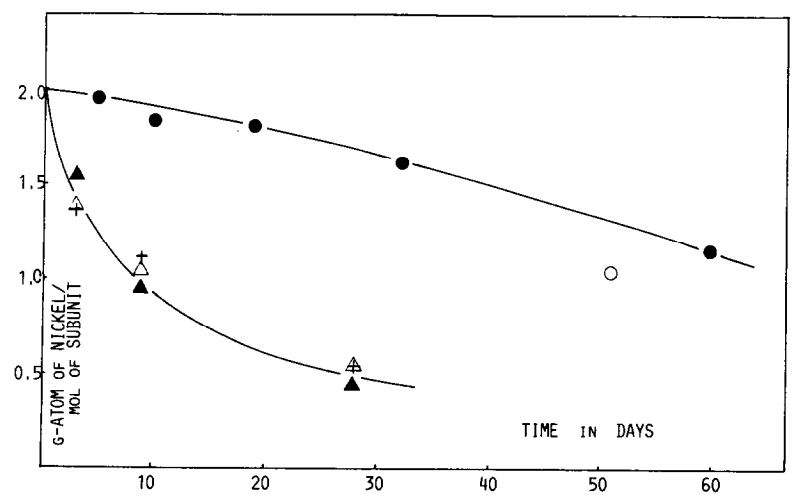

FIG. 2. Time course for the loss of nickel from jack bean urease at 25°C: (●) Dialysis in 0.05 M MES buffer (pH 7.0, 0.2 mM in Na_2SO_3); (○) dialysis in 0.05 M MES buffer (pH 7.0, 0.2 mM in Na_2SO_3, 1 mM in oxalic acid); (+) dialysis in 0.1 M NEM buffer, pH 7.0; (△) dialysis in 0.1 M NEM buffer (pH 7.0, 1.0 mM in oxalic acid); (▲) dialysis in 0.1 M NEM buffer (pH 7.0, 1.0 mM in EDTA).

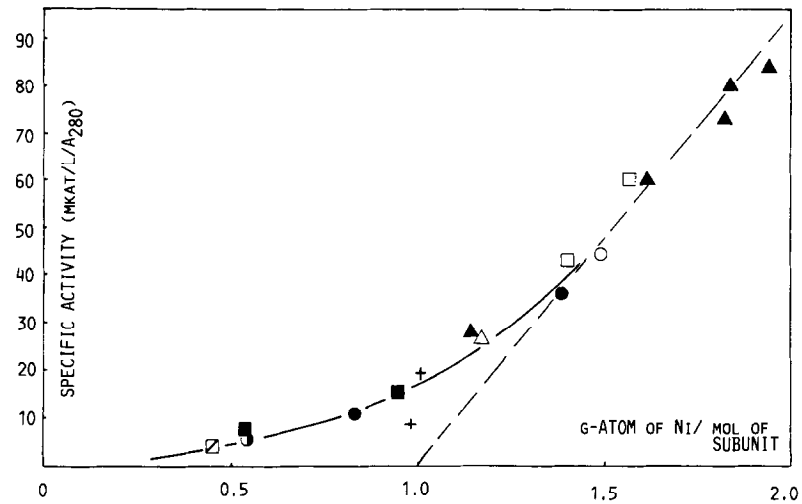

FIG. 3. Changes in specific activity which occur with the loss of nickel from jack bean urease at 25°C: (▲) Dialysis in 0.05 M MES buffer (pH 7.00, 0.2 mM in Na_2SO_3), 5, 10, 21, 32, and 60 days; (□) dialysis in 0.05 M MES buffer (pH 7.0, 1.0 mM in DTT, 1.0 mM in oxalic acid), 14 and 20 days; (○) on standing in 0.05 M MES buffer (pH 7.0, 0.2 mM in Na_2SO_3), 60 days; (●) dialysis in 0.05 M MES buffer (pH 7.0, 0.2 mM in Na_2SO_3, 1.0 mM in oxalic acid, 0.1 mM in $ZnSO_4$), 20 and 53 days; (△) dialysis in 0.05 M MES buffer (pH 7.0, 0.2 mM in Na_2SO_3, 1.0 mM in oxalic acid, 0.1 mM in $Co(NO_3)_2$), 60 days; (+) dialysis in 0.05 M MES buffer (pH 7.0, 0.2 mM in Na_2SO_3, 1.0 mM in oxalic acid), 51 and 82 days; (■) dialysis in 0.1 M NEM buffer (pH 7.0, 1.0 mM in EDTA), 9 and 28 days; (◑) dialysis in 0.1 M NEM buffer (pH 7.0, 1.0 mM in oxalic acid), 28 days; (⊠) dialysis in 0.1 M NEM buffer, pH 7.0, 28 days.

nickel have met with very limited success. Recently, however, we have begun to define a system which has already yielded useful data, and which with further modification will no doubt enable us to make significant headway in this important area (*81*).

The definition of the system depends on the interaction of urease with sulfite. Urease is absolutely stable in the presence of relatively high concentrations of sulfite (20–50 mM), and we conventionally store the enzyme in buffered 50 mM sulfite. If, however, the sulfite is completely removed from the protein, nickel ion is lost on a reasonable time scale. The principal observations are summarized in Fig. 2 about which the following points may be made.

The lower curve, which indicates a relatively rapid loss of nickel in 0.1 M *N*-ethylmorpholinium (NEM) buffer at pH 7.0 in the absence of sulfite, also shows that the rate of this loss is not enhanced by either 1.0 mM oxalate or 1.0 mM EDTA. What Fig. 2 does not reveal is that during the time required to lose 75% of the nickel, some 90% of the urease protein has irreversibly precipitated. The upper curve shows that the nickel is more slowly lost in the presence of 0.2 mM sulfite in a 0.05 M morpholinium ethanesulfonate (MES) buffer, but under these conditions, the protein remains completely soluble. Further, the electrophoretic and ultracentrifugal properties of the nickel-depleted enzyme are the same as those of the native enzyme. These are the basic conditions which have enabled us

to investigate how the specific activity is affected by the loss of nickel ion and by the replacement of nickel with other metal ions.

Figure 3 summarizes a mass of data relating to nickel loss and replacement. At the outset, it should be stressed that these data have been acquired over varying periods of time (from 5 to 82 days) and under a variety of conditions (see legend for Fig. 3). Nonetheless, the tangential dashed line indicates that the loss of one nickel ion should result in an enzyme which has zero activity. This situation is not realized in practice presumably because the residual nickel can reequilibrate between the two sites as discussed earlier. Included in Fig. 3 are cobalt- and zinc-substituted ureases, but the data are such that at this stage it is not possible to decide whether the substituted enzymes have any activity of their own. Thus the cobalt-substituted sample, produced by dialysis of the native enzyme in the presence of 0.1 mM Co^{2+} for 60 days, was found to contain 1.17 g-atom of nickel and 0.66 g-atom of cobalt per 96.6-kDa subunit. The specific activity of this sample was 25.4 mkat/liter/A_{280}. The zinc-substituted enzyme, similarly produced after 53 days, contained 0.89 g-atom of nickel and 0.83 g-atom of zinc. This sample had a specific activity of 10.5 mkat/liter/A_{280}. These measurements are made uncertain because we do not have secure knowledge of the ultraviolet spectra of the substituted enzymes. Suffice that we have here a system which can now be exploited for metal substitutions.

Subsequent work has shown that the enzyme is reasonably stable in buffered 2 μM sulfite, and that the nickel is lost still more rapidly.

CONCLUSION

Far from providing little excitement for me—and I hope those students who desperately tried to come to grips with the enzyme—the 20-odd years of this study of urease have provided a theatre for the scrutiny of the very essence of enzymatic catalysis. The concept of substrate specificity has been reshaped in large part as a result of work on this enzyme's reactivity. Our understanding of the role of metal ions in enzymatic catalysis and means for the determination of their involvement in this process have been further developed. Most recently, the enzyme has served as a vehicle for the investigation of the relatedness of enzymes from different phylogenetic sources through the application of DNA technology, and for a more thorough description of the interactions of the nickel and other ions with the enzyme protein. The beautiful work of Berg and Merkle on the metal ion specificity of the "zinc finger" proteins is apposite in this latter regard (*82*). All of this has been achieved with no help from X-ray crystallography, because the well-known octahedral crystals of the enzyme diffract poorly (*83*). We are currently preparing derivatives of the protein to attempt to obtain other crystalline forms which might be more conducive to X-ray structure determination (*84*).

Finally, this article will be an appropriate tribute to Myron Bender if it leaves the reader with just some small measure of the joy of good graduate students and of the excitement I have known.

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REFERENCES

1. GREGORY, H. (1987) Vivant Professores, Fryer Memorial Library, Occasional Paper No. 7, pp. 68–71, University of Queensland, St Lucia.
2. SUMNER, J. B. (1926) *J. Biol. Chem.* **69,** 435.
3. VAN TIEGHEM, M. (1864) *C. R. Acad. Sci.* **58,** 210.
4. MUSCULUS, F. (1874) *C. R. Acad. Sci.* **78,** 132.
5. MUSCULUS, F. (1876) *C. R. Acad. Sci.* **82,** 333.
6. Encyclopaedia Britannica (1929) pp. 630–631, Encyclopaedia Britannica, Chicago.
7. WILLSTÄTTER, R. (1949) Aus Meinem Leben, p. 363, Verlag Chemie, Weinheim.
8. WILLSTÄTTER, R. (1933) *Chem. Rev.* **13,** 501.
9. ROBINSON, R. (1953) *J. Chem. Soc.* 999.
10. BLAKELEY, R. L., WEBB, E. C., AND ZERNER, B. (1969) *Biochemistry* **8,** 1984.
11. BLAKELEY, R. L., HINDS, J. A., KUNZE, H. E., WEBB, E. C., AND ZERNER, B. (1969) *Biochemistry* **8,** 1991.
12. The 1969 analysis of our then purest sample of urease which had been dialyzed into oxygen-free distilled water and then lyophilized, gave as metals present in significant amount Ca > Al > Ni > Fe > Zn. Since Ca and Al were reported by Vallee to be common contaminants of many proteins, since all amounts showed no simple relationship to the Sumner M_r of 483,000, and since the analysis for nickel was very low, we erroneously accepted Vallee's proposition that the enzyme was not a metalloenzyme.
13. MERTZ, W. (1970) *Fed. Proc.* **29,** 1482.
14. NIELSEN, F. H. (1984) *Annu. Rev. Nutr.* **4,** 21.
15. DIXON, N. E., GAZZOLA, C., WATTERS, J. J., BLAKELEY, R. L., AND ZERNER, B. (1975) *J. Amer. Chem. Soc.* **97,** 4130.
16. DIXON, N. E., GAZZOLA, C., BLAKELEY, R. L., AND ZERNER, B. (1975) *J. Amer. Chem. Soc.* **97,** 4131.
17. BARTHA, R., AND ORDAL, E. J. (1965) *J. Bacteriol.* **89,** 1015.
18. FRIEDRICH, B., HEINE, E., FINCK, A., AND FRIEDRICH, C. G. (1981) *J. Bacteriol.* **145,** 1144.
19. CAMMACK, R., FERNANDEZ, V. M., AND SCHNEIDER, K. (1988) *in* The Bioinorganic Chemistry of Nickel (Lancaster, J. R., Jr., Ed.), Chap. 8, VCH, New York.
20. MOURA, J. J. G., TEIZEIRA, M., MOURA, I., AND LEGALL, J. (1988) *in* The Bioinorganic Chemistry of Nickel (Lancaster, J. R., Jr., Ed.), Chap. 9, VCH, New York.
21. BASTIAN, N. R., WINK, D. A., WACKETT, L. P., LIVINGSTON, D. J., JORDAN, L. M., FOX, J., ORME-JOHNSON, W. H., AND WALSH, C. T. (1988) *in* The Bioinorganic Chemistry of Nickel (Lancaster, J. R., Jr., Ed.), Chap. 10, VCH, New York.
22. FRIEDRICH, C. G., SCHNEIDER, K., AND FRIEDRICH, B. (1982) *J. Bacteriol.* **152,** 42.
23. GRAF, E. G., AND THAUER, R. K. (1981) *FEBS Lett.* **136,** 165.
24. DIEKERT, G. B., GRAF, E. G., AND THAUER, R. K. (1979) *Arch. Microbiol.* **122,** 117.
25. DRAKE, H. L., HU, S. I., AND WOOD, H. G. (1980) *J. Biol. Chem.* **255,** 7174.

26. ELLEFSON, W. L., AND WOLFE, R. S. (1981) *J. Biol. Chem.* **256,** 4259.
27. ELLEFSON, W. L., WHITMAN, W. B., AND WOLFE, R. S. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 3707.
28. NIEBOER, E., TOM, R. T., AND SANFORD, W. E. (1988) *in* Metal Ions in Biological Systems (Sigel, H., Ed.), Chap. 4, M. Dekker, New York.
29. ANKEL-FUCHS, D., AND THAUER, R. K. (1988) *in* The Bioinorganic Chemistry of Nickel (Lancaster, J. R., Jr., Ed.), Chap. 5, VCH, New York.
30. MOURA, J. J. G., MOURA, I., TEIXEIRA, M., XAVIER, A. V., FAUQUE, G. D., AND LEGALL, J. (1988) *in* Metal Ions in Biological Systems (Sigel, H., Ed.), Chap. 7, M. Dekker, New York.
31. WACKETT, L. P., HONEK, J. F., BEGLEY, T. P., SHAMES, S. L., NIEDERHOFFER, E. C., HAUSINGER, R. P., ORME-JOHNSON, W. H., AND WALSH, C. T. (1988) *in* The Bioinorganic Chemistry of Nickel (Lancaster, J. R., Jr. Ed)., Chap. 11, VCH, New York.
32. PFALTZ, A. (1988) *in* The Bioinorganic Chemistry of Nickel (Lancaster, J. R., Jr., Ed.), Chap. 12, VCH, New York.
33. DIEKERT, G. (1988) *in* The Bioinorganic Chemistry of Nickel (Lancaster, J. R., Jr., Ed.), Chap. 13, VCH, New York.
34. RAGSDALE, S. W., WOOD, H. G., MORTON, T. A., LJUNGDAHL, L. G., AND DERVARTANIAN, D. V. (1988) *in* The Bioinorganic Chemistry of Nickel (Lancaster, J. R., Jr., Ed.), Chap. 14, VCH, New York.
35. ANDREWS, R. K., BLAKELEY, R. L., AND ZERNER, B. (1988) *in* The Bioinorganic Chemistry of Nickel (Lancaster, J. R., Jr., Ed.), Chap. 7, VCH, New York.
36. WERNER, E. A. (1923) The Chemistry of Urea, Longmans, London.
37. WARNER, R. C. (1942) *J. Biol. Chem.* **142,** 705.
38. SHAW, W. H. R., AND BORDEUX, J. J. (1955) *J. Amer. Chem. Soc.* **77,** 4729.
39. SHAW, W. H. R., AND WALKER, D. G. (1958) *J. Amer. Chem. Soc.* **80,** 5337.
40. WILLIAMS, A., AND JENCKS, W. P. (1974) *J. Chem. Soc., Perkin Trans. 2* 1753.
41. WILLIAMS, A., AND JENCKS, W. P. (1974) *J. Chem. Soc., Perkin Trans. 2* 1760.
42. DITTERT, L. W., AND HIGUCHI, T. (1963) *J. Pharm. Sci.* **52,** 852.
43. BENDER, M. L., AND HOMER, R. B. (1965) *J. Org. Chem.* **30,** 3975
44. FITZGERALD, L. R., BLAKELEY, R. L., AND ZERNER, B. (1984) *Chem. Lett.* 29.
45. DIXON, N. E., RIDDLES, P. W., GAZZOLA, C., BLAKELEY, R. L., AND ZERNER, B. (1980) *Canad. J. Biochem.* **58,** 1335.
46. WOOLLEY, E. M., AND HEPLER, L. G. (1974) *Anal. Chem.* **44,** 1520.
47. ANDREWS, R. K. (1986) Ph.D. thesis, University of Queensland, Brisbane.
48. FISHBEIN, W. N., WINTER, T. S., AND DAVIDSON, J. D. (1965) *J. Biol. Chem.* **240,** 2402.
49. FISHBEIN, W. N., AND CARBONE, P. P. (1965) *J. Biol. Chem.* **240,** 2407.
50. MAMIYA, G., TAKISHIMA, K., MASAKUNI, M., KAYUMI, T., OGAWA, K., AND SEKITA, T. (1985) *Proc. Japan Acad., Ser. B* **61,** 395.
51. ANDREWS, R. K., BLAKELEY, R. L., AND ZERNER, B. (1988) *in* Metals Ions in Biological Systems (Sigel, H., Ed.), Vol. 23, Chap. 6, p. 258, M. Dekker, New York.
52. TAKISHIMA, K., SUGA, T., AND MAMIYA, G. (1988) *Eur. J. Biochem.* **175,** 151.
53. DIXON, N. E., HINDS, FIHELLY, A. K., GAZZOLA, C., BLAKELEY, R. L., AND ZERNER, B. (1980) Canad. J. Biochem. **58,** 1323.
54. GAZZOLA, C. (1976) Ph.D. thesis, University of Queensland, Brisbane.
55. DIXON, N. E., BLAKELEY, R. L., AND ZERNER, B. (1980) *Canad. J. Biochem.* **58,** 469.
56. HAMILTON, S., AND ZERNER, B. (1982) *J. Amer. Chem. Soc.* **103,** 1827.
57. WESTHEIMER, F. H. (1970) *Search* **1,** 34.
58. KOKESH, F. C., AND WESTHEIMER, F. H. (1971) *J. Amer. Chem. Soc.* **93,** 7270.
59. The tyrosine residue is set in italic as the most likely residue because there is no evidence for Cys ligation to the nickel ion under normal conditions, and Asp and Glu residues would need to have abnormally low dissociation constants if they are to become fully protonated.
60. ANDREWS, R. K., BLAKELEY, R. L., AND ZERNER, B. (1975) *in* Advances in Inorganic Biochemistry (Eichhorn, G. L., and Marzilli, L. G., Eds.), Vol. 6, Chap. 7, Academic Press, New York.
61. BLAKELEY, R. L., DIXON, N. E., AND ZERNER, B. (1983) *Biochim. Biophys. Acta* **744,** 219.
62. BLAKELEY, R. L., AND ZERNER, B. (1984) *J. Mol. Catal.* **23,** 263.

63. ANDREWS, S. K., DEXTER, A., BLAKELEY, R. L., AND ZERNER, B. (1986) *J. Amer. Chem. Soc.* **108,** 7124.
64. KOBASHI, K., SAKAGUCHI, K., TAKEBE, S., HASE, J., AND SATO, M. (1982) *Abstr. Int. Congr. Biochem., Perth, 12th* 305.
65. See, for example: Dayhoff, M. O. (Ed.) (1976) Atlas of Protein Sequence and Structure, Chap. 4, pp. 25–49, and Chap. 12, pp. 191–223, Natl. Biomed. Res. Foundation, Washington, D.C.
66. RIDDLES, P. W., ANDREWS, R. K., BLAKELEY, R. L, AND ZERNER, B. (1983) *Biochim. Biophys. Acta* **43,** 115.
67. LILJAS, A., AND ROSSMANN, M. G. (1974) *Annu. Rev. Biochem.* **43,** 475.
68. Ref. 51, Table 8 and Paragraph 9.4.
69. The Japanese workers commonly call the jack bean the sword bean. This presumably derives from the species name *Canavalia ensiformis,* where *ensiformis* means "sword shaped."
70. SAKAGUCHI, K., MITSUI, K., KOBASHI, K., AND HASE, J. (1983). *J. Biochem. (Tokyo)* **93,** 681.
71. Independent evidence for the involvement of histidine at the active site of this enzyme has been obtained in this laboratory (Andrews, R. K., and Zerner, B., unpublished results).
72. WHAN, V. A., BLAKELEY, R. L., ZERNER, B., AND RIDDLES, P. W. (1990) *Proc. Aust. Biochem.* **22,** P24.
73. PEMBERTON, J. M., AND WALKER, M. J. (1988), unpublished results.
74. KIM, S. D., AND SPITIZIZEN, J. (1985) *Korean J. Appl. Microbiol. Bioeng.* **13,** 297.
75. TODD, M. J., AND HAUSINGER, R. P. (1987) *J. Biol. Chem.* **262,** 5963.
76. BLAKELEY, R. L., KIM, S. D., PEMBERTON, J. M., PHAM, V. A., WALKER, M. J., WHAN, V. A., ZERNER, B., AND RIDDLES, P. W. (1990) *Proc. Aust. Biochem. Soc.* **22,** P35a.
77. DE BOER, H. A., AND KATELEIN, R. A. (1986) *in* Maximizing Gene Expression (Reznikoff, W., and Gold, L., Eds.), Chap. 8, Butterworth, Stoneham, MA.
78. IKEMURA, T., AND OZEKI, H. (1982) *Cold Spring Harbor Symp. Quant. Biol.* **47,** 1087.
79. MOBLEY, H. L. T., AND HAUSINGER, R. P. (1989) *Microbiol. Rev.* **53,** 85.
80. DIXON, N. E., GAZZOLA, C., ASHER, C. J., LEE, D. S. W., BLAKELEY, R. L., AND ZERNER, B. (1980) *Canad. J. Biochem.* **58,** 474.
81. KING, G. J., AND ZERNER, B. (1989), unpublished results.
82. BERG, J. M., AND MERKLE, D. L. (1989) *J. Amer. Chem. Soc.* **111,** 3759.
83. COLMAN, P. M., FREEMAN, H., QUIOCHO, F., AND ZERNER, B. (1975–1989), unpublished results.
84. BALLS, A. K., MCDONALD, C. E., AND BRECHER, A. S. (1958) Proceedings of the International Symposium on Enzyme Chemistry, p. 392, Maruzen, Tokyo.